MILK GENOMICS

VARIATION IN MILK COMPOSITION FROM MAJOR DANISH DAIRY BREEDS AND EXPLOITATION INTO DAIRY PRODUCTS

NINA AAGAARD POULSEN, ULRIK SUNDEKILDE, IDA EMILIE INGVORDSEN LINDAHL, BART BUITENHUIS AND LOTTE BACH LARSEN

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Variation in milk composition in major Danish dairy breeds and exploitation in dairy products

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Data sheet

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0. Preface

The aim of the present report is to document levels and natural variability in the composition of Danish dairy milk. The results are from the Danish Milk Genomics Initiative as well as from subsequent projects related thereto. The original Danish Milk Genomics Initiative was launched in 2009 based on funding from Arla Foods amba, The Danish Milk Levy Fund, Aarhus University and a larger grant from the Innovation Fund Denmark, which enabled initiation of the large sampling. During the execution of the initiative, the initial research grants were supplemented with additional research grants from various sources, as listed in the original peer-reviewed papers. The core Milk Genomics was carried out in close collaboration with Sweden, also known collaboratively as the Danish-Swedish Milk Genomics Initiative. The results reported here primarily relate to the analyses of Danish milk and represent mainly data from the core project in Denmark, carried out from 2009-2014, but overall of course reflect outcomes of a very fruitful collaboration with Lund University and Swedish Agricultural University in Sweden. The main Milk Genomics core project bio-material comprised samplings of milk and DNA from individual cows and elucidation of the milk compositional, technological and nutrition related traits in relation to breed, herd, genetics and parity in healthy, conventional dairy cows in mid-lactation, with the further aim to relate this information to its potential exploitation in the dairy chain. It was chosen that this report comprises the two Danish breeds included in the Milk Genomics studies, and not results from Swedish Red unless it fits into the connection. The hope is, that this detailed report can provide sufficient information about Danish milk quality for future benchmarking of the milk composition of the two Danish dairy breeds, Danish Holstein and Danish Jersey as well as being a useful table material for documentations of variations in Danish dairy milk at cow level.

The foundation for the Milk Genomics Initiative was laid by combining the emerging of new “omics” technologies (genomics, metabolomics and proteomics) alongside with observed natural variation and search for explanations. We want to thank all the participants in these milk genomics studies, from participating farmers, technicians, students, scientists involved, as well as our industry collaborators at Arla Foods amba and Viking Genetics for their support and discussions, as well as specifically Thise Dairy and Arla Foods amba for a special grant enabling this Danish Milk Genomics knowledge synthesis to be done.

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1. Introduction

Milk is an important source of nutrients, and is recommended to be part of an everyday, balanced diet. Milk contains a wide range of nutrients, among others saturated and unsaturated fatty acids, proteins, carbohydrates (mainly lactose), minerals and vitamins, which in their natural or manufactured forms potentially promote positive health effects. Consumption of milk and dairy products can thereby not only provide nutrients, but also potentially exert protective effects on human health (Haug et al., 2007; Givens, 2010). A schematic flow of the milk phenotypes measured in the Milk Genomics project is given in Figure 1.

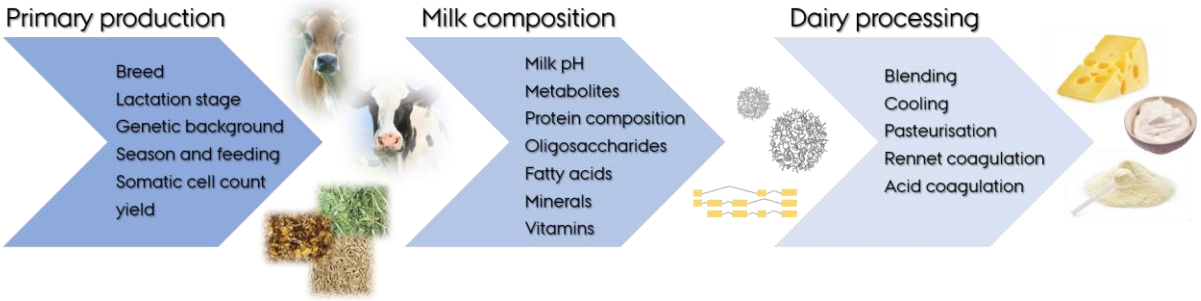


Figure 1. Flow of traits and parameters covered by the study.

The Danish-Swedish Milk Genomics Initiative has resulted in more than 40 international publications exploring natural variation in milk components by implementation of new methodologies like metabolomics and proteomics, in relation to genetic influence of measured traits. A list of most relevant publications reporting on levels of measured parameters coming from the milk genomics project and of most relevance for this report is given in Appendix 1.

Sample collection and overall milk composition

As a part of the Danish Milk Genomics initiative samples were collected from 456 Danish Holstein cows (20 dairy herds, October–December 2009) and from 436 Danish Jersey cows (22 dairy herds, February–April 2010). Sampling was carried out at one morning milking at conventional (nonorganic) herds, in-door housing and with no automatic milking. The cows were primarily milked twice daily and only rarely 3 times.

The overall experimental strategy underlying the study was to minimize potential sources of environmental variation while maximizing genetic variation in the sample population. As a result, the pedigree of the selected animals was designed to include as unrelated animals as possible (i.e. maximizing the number of sires). Genomic DNA was extracted from collected ear tissue. Cows were genotyped using the bovineHD beadchip (Illumina) and in total 777,962 SNP markers were assayed.

Milk yield at the particular morning milking was recorded and representative milk samples of at least 0.5 L were placed on ice during transport to the laboratory. All animals were in mid-lactation (week 18-36) and within the first three parities (Table 1). Herd-specific feeding plans were provided by the dairy farmers and relative feed proportions of grain, maize silage, grass products (mainly grass silage, but also minor amounts of whole crop silage, hay, and straw), and concentrate were calculated (Table 1). These broad feed categories were defined to comprise the actual different feed sources used in the herds, and which may influence in particular the fatty acid composition. The experimental design enabled that the genetic effect on trait variability could be disentangled from environmental/management effect (Figure 2).

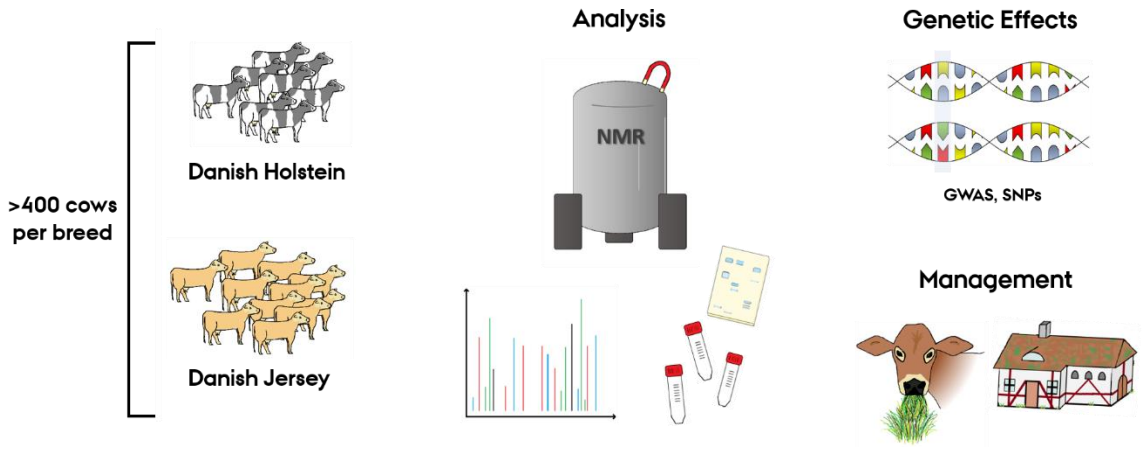


Figure 2. The aim of the Milk Genomics Initiative was to elucidate the effect of breed, herd, genetics and parity on the composition of milk from the conventional breeds Danish Holstein and Danish Jersey.

Milk yield, lactose content, SCC, pH and conductivity were significantly higher in DH compared to DJ (Table 1). In contrast milk from DJ cows was more concentrated and had a significantly higher content of fat and protein (Table 1).

Table 1. Descriptive statistics of parity, days in milking (DIM), somatic cell count (SCC) and overall milk composition in 456 Danish Holstein (20 herds) and 435 Danish Jersey (22 herds). Adapted from Poulsen et al. (2012).

	Danish Holstein			Danish Jersey		
Trait[1]	Mean	Min – max	CV%	Mean	Min - max	CV%
Parity	1.73	1-3	44.48	1.73	1-3	45.00
DIM	179.5[a]	129-228	11.95	185.9[b]	130-252	12.3
Milk yield (kg)*	14.6[a]	3.0-26.5	26.8	10.1[b]	2.0-19.0	26.1
Fat % (g/100 g)	4.02[a]	1.52-9.20	20.32	5.99[b]	3.31-11.57	14.46
Protein % (g/100 g)	3.44[a]	2.82-4.31	7.67	4.30[b]	3.34-5-45	7.45
Casein % (g/100 g)	2.66[a]	2.36-3.05	4.64	3.02[b]	2.51-3.55	4.99
Lactose % (g/100 g)	4.78[a]	4.09-5.09	3.10	4.62[b]	3.58-4.94	3.30
SCC (*1000 cells/mL)	204[a]	4-5300	221	189[b]	4-7878	271
pH	6.69[a]	6.50-6.98	1.04	6.67[b]	6.42-6.88	0.89
Conductivity (mS/cm)	5.84[a]	4.64-7.67	7.26	5.44[b]	4.10-6.88	7.55
Grass products %**	21[a]	11-31	22	20[a]	8-31	31
Maize silage %**	38[a]	29-49	14	34[a]	26-47	17
Grain %**	7[a]	0-14	84	8[a]	0-23	93
Concentrate %**	34[a]	21-50	20	39[a]	26-53	15

Different superscript letters within a row indicate significant ($p<0.05$) differences between means.
*Morning milk yield
**At herd level

1.1 Fatty acids

Breed differences

The fatty acid compositions of Danish Holstein and Danish Jersey milk are presented in table 2. In total, 17 individual fatty acids were determined by gas chromatography and evaluated together with calculated n-3/n-6 ratio and desaturase indices (Poulsen et al., 2012). The desaturase indices were calculated as the ratio between product and sum of product and substrate, and used as a proxy for Δ9-desaturase activity. In line with other studies (Hermansen and Lund, 1990; White et al., 2001; Larsen et al., 2012), higher levels of short- and (to some extent) medium-chain fatty acids (C6-C12), as well as lower level of unsaturated fatty acids were found in Danish Jersey compared with Danish Holstein. The higher level of C6-C12 suggest a higher *de novo* fatty acid synthesis in the mammary gland in Danish Jersey compared to Danish Holstein. The desaturase indices (C14, C16, C18 and CLA indices, Table 2), calculated using product to substrate ratio as a proxy for desaturase activity, were generally higher in milk from Danish Holstein cows, indicating a lower genuine desaturase activity in Danish Jersey. Especially, the C14 index should be a good measure of the desaturase activity, as C14:0 derives almost solely from the *de novo* synthesis within the mammary gland, thus C14:1 should purely be synthesised in the mammary gland (Peterson et al., 2002). The observed differences between Danish Holstein and Danish Jersey are in accordance with other studies (DePeters et al., 1995; Carroll et al., 2006).

Effect of feed

The interplay between the content and composition of fat in feed and the composition of milk fat is rather complex, with multiple effects on the fatty acids composition, depending on e.g. level and choice of feed source and forage-to-concentrate ratio. Generally, the content of saturated fatty acids in milk is high, but increasing addition of fat to the diet reduces the *de novo* synthesis in the mammary gland and thus the content of saturated short- and medium-chain fatty acids in the milk fat (Grummer, 1991). Due to rumen hydrogenation, the transfer of unsaturated fatty acids from feed to milk is relatively low (Jenkins and McGuire, 2006). Thus, the ability to predict the content of individual fatty acids from the composition of dietary fat varies among fatty acids (Hermansen, 1995).

Within breeds, the mammary gland *de novo* synthesised fatty acids were generally highly positively correlated with each other and negatively correlated with C16:0 and C18 fatty acids, in line with the earlier reported results (Karijord et al., 1982). This pattern clearly reflects the common origin of different fatty acids based on the *de novo*-synthesised fatty acids, the feed-derived fatty acids and those being primarily regulated by the desaturase activity (C14:1 and C16:1).

Based on information from the herd-specific feeding plans the feeding regimens were found to have a significant effect on the fatty acid composition of both breeds. The amount of maize silage in feed was negatively correlated to C16:0 and C16:1, respectively, and positively correlated to the contents of C18:1 *trans*-11 and CLA *cis*-9, *trans*-11. This pattern was mainly associated with maize silage being a significant source of C18:2 n-6, which is hydrogenated to C18:1 *trans*-11 (Slots et al., 2009). A negative correlation was found between grass feeding and C18:2 cis-9,12 (linoleic acid) content in the milk. The n-3:n-6 ratio was positively correlated to a feeding regime that included grass products. The n-3:n-6 ratio in milk from Danish Jersey cows was negatively affected by grain. Finally, concentrate feeding was also found to affect the fatty acid composition in both breeds, as positive correlations were observed for C18:1 trans-11 in Danish Jersey and for C18:1 cis-9 in Danish Holstein, respectively (Poulsen et al., 2012). Variance components were estimated and used to determine the proportion of phenotypic variation that could be explained by herd. The herd effect for individual fatty acids was generally lower for Danish Holstein. In addition, very low herd effects were shown for C14:1 and C16:1 in both breeds, suggesting that the content of these fatty acids is mainly genetically regulated (Poulsen et al., 2012).

Table 2. Fatty acids and related traits in Danish Holstein and Danish Jersey presented as mean, min, max and coefficient of variation (CV%). Fatty acids are given in g/kg fatty acids. The table is modified from Poulsen et al. (2012).

	Danish Holstein			Danish Jersey		
Trait[1]	Mean	Min – max	CV%	Mean	Min – max	CV%
C6:0	26.83[a]	15.89-36.28	12.82	27.94[b]	19.93-38.59	10.69
C8:0	14.62[a]	7.54-20.39	15.44	16.15[b]	9.21-23.88	11.63
C10:0	31.52[a]	15.75-45.95	18.02	35.61[b]	15.90-52.21	13.61
C12:0	35.62[a]	20.01-53.17	18.39	40.56[b]	18.09-61.07	15.06
C13:0	1.00[a]	0.32-2.41	30.79	1.38[b]	0.37-4.77	33.80
C14:0	112.34[a]	68.90-144.76	11.46	105.80[b]	63.46-135.65	8.48
C14:1	9.73[a]	2.83-22.76	28.06	8.32[b]	4.03-14.22	20.54
C15:0	10.96[a]	6.73-18.43	17.69	11.91[b]	5.17-19.50	17.31
C16:0	289.13[a]	205.70-400.85	11.54	303.72[b]	220.74-399.62	10.89
C16:1	15.02[a]	3.85-31.01	25.40	13.80[b]	3.44-30.90	21.97
C17	5.27[a]	0.60-9.77	27.94	5.20[a]	3.33-9.30	20.75
C18:0	104.50[a]	58.66-209.27	19.48	116.78[b]	49.09-179.16	14.50
C18:1 *cis*-9	200.75[a]	2.90-55.31	30.87	164.23[b]	4.72-32.92	26.83
C18:1 *trans*-11[2]	16.84[a]	123.59-292.15	14.51	15.05[b]	119.17-280.92	12.45
C18:2 *cis*-9, 12	16.94[a]	10.05-28.53	16.89	15.18[b]	8.86-35.84	19.87
C18:2 *cis*-9, *trans*-11	6.28[a]	3.10-12.03	24.84	4.49[b]	2.12-9.56	25.66
C18:3 *cis*-9, 12, 15	4.93[a]	2.38-7.62	20.51	4.07[b]	1.63-7.33	18.74
C6-C10	72.97[a]	40.49-100.72	14.76	79.70[b]	47.73-108.21	11.00
C12-C14	148.95[a]	92.42-199.10	12.59	147.74[a]	86.43-194.71	9.68
Ratio n-3/n-6 (%)[3]	29.33[a]	14.87-46.24	17.56	27.35[b]	11.74-44.24	19.25
C14 index (%)[4]	7.95[a]	2.87-17.15	24.49	7.28[b]	3.59-11.96	18.21
C16 index (%)[5]	4.93[a]	1.34-9.79	21.46	4.35[b]	0.95-9.97	18.60
C18 index (%)[6]	65.80[a]	45.37-77.47	6.53	58.49[b]	50.82-76.42	5.57
CLA index (%)[7]	27.78[a]	12.30-71.31	21.65	23.23[b]	14.15-46.65	14.14

[a-b]Significant differences among breeds ($P < 0.05$).
[1]Fatty acids were determined by gas chromatography and expressed as weight proportion of total identified FA according to Larsen et al., (2011).
[2]C18:1 trans-11: mixture of C18:1 trans-11 and C18:1 trans-10.
[3]Ratio n-3/n-6 = (C18:3 cis-9, 12, 15/C18:2 cis-9, 12) × 100.
[4]C14 index = C14:1/(C14:1 + C14:0) × 100.
[5]C16 index = C16:1/(C16:1 + C16:0) × 100.
[6]C18 index = C18:1 cis-9/(C18:1 cis-9 + C18:0) × 100.
[7]CLA index = C18:2 cis-9, trans-11/ (CLA cis-9, trans-11 + C18:1 trans-11) × 100.

1.2. Major milk proteins

The major milk proteins are heterogenous due to genetic polymorphisms leading to amino acid changes in the peptide backbone or deletions, and furthermore many have posttranslational modifications (PTM), including phosphorylations and glycosylations. Several genetic variants of the major milk proteins have been identified in cattle (Farrell et al., 2004; Caroli et al., 2009). In addition, phosphate groups are esterified to the casein (CN) molecules during synthesis, via hydroxyl groups of mainly serine residues, making P-Ser. These phosphorylated serines are anchor points for the micellar bound calcium (colloidal calcium phosphate). The number of phosphorylations commonly found are in the ranges of: α_{S1}-CN (8-9P), α_{S2}-CN (10-13P) and β-CN (5P).In addition κ-CN normally contains 1P

(0-3 seem to occur, 95% of the molecules are phosphorylated; Jensen et al. 2012). In Figure 3, all possible sites for the natural PTMs of the CNs are illustrated, i.e. the maximal number of phosphorylation and glycosylation sites that have been reported for each protein in order to illustrate all potential positions of these modifications. Not all sites are in use on all molecules (for details, see Le et al., 2017), and therefore the major form found in milk of each type is indicated (Figure 3). Important structural features of the major milk proteins are further summarized in Table 3.

In addition to phosphorylations, κ-CN molecules are glycosylated (Figure 3). The O-glycosylation of k-CN results in glycan attachment at threonine residues, resulting in attachment of 1 to 6 glycans at the specific sites: Thr142, Thr152, Thr154, Thr157 (only variants A and E), Thr163, Thr166, Thr186. Around 30-40% of the κ-CN molecules (27-46 % in individual cow's milk) have been estimated to be glycosylated, and thereby 60-70 % is non-glycosylated (Jensen et al., 2015a). In addition, there are two cysteine residues (Cys11 and Cys88) in κ-CN and two cysteine in α_{s2}-CN (Cys36 and Cys40). By addition of chymosin (rennet), the k-CN, which is situated on the surface of the CN micelles, will be hydrolysed at Phe105-Met106 into caseino-macropeptide (CMP) and para-k-CN, resulting in milk coagulation, which is the first step in cheese manufacturing.

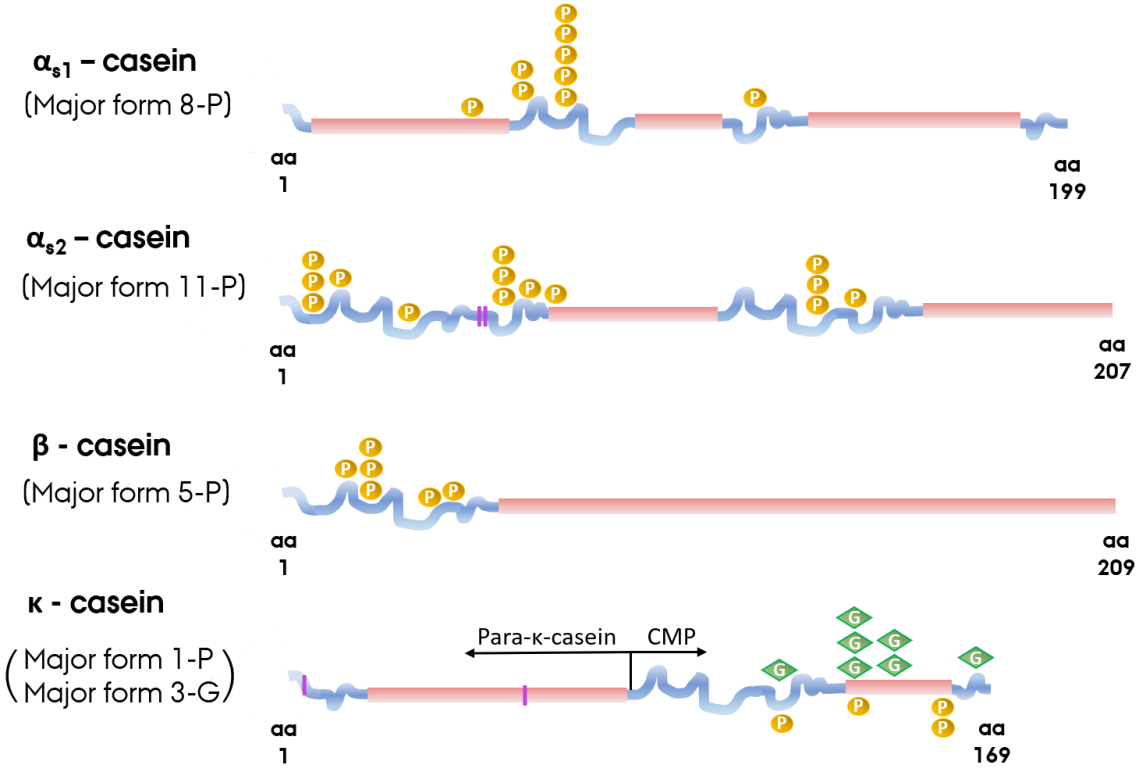


Figure 3. Post translational modifications of caseins based on the review by Le et al. (2017). Hydrophilic and hydrophobic regions of the mature protein are indicated. Blue stretches: hydrophilic regions, red stretches: hydrophobic regions. Purple lines indicate positions of Cys. All possible phosphorylation and glycosylation sites are highlighted. P: phosphorylation, G: glycosylation. Note that stacked phosphorylations and glycosylation are due to PTM sites in close proximity on the protein, which due to scale appears to be at similar positions. Figure from Poulsen & Larsen (2022).

In contrast to the CNs, the major whey proteins; α-lactalbumin and β-lactoglobulin are not phosphorylated, and only α-lactalbumin can be glycosylated (N-glycosylated at Asn45), but with the major form being non-glycosylated (Farell et al., 2004). Both these major whey proteins contain cysteines, and have disulphide bonds: α-lactalbumin has cysteine at positions 6, 28, 61, 73, 77, 91, 111 and 120, which are all engaged in disulphide bonds (4); β-lactoglobulin has cysteine at positions 66, 106, 119, 121 and 160 (Figure 4), forming 2 disulphide bonds and with Cys121 present natively as a free Cys, but which can interact and promote disulphide interchanges and multimerization at various conditions, including heat treatment.

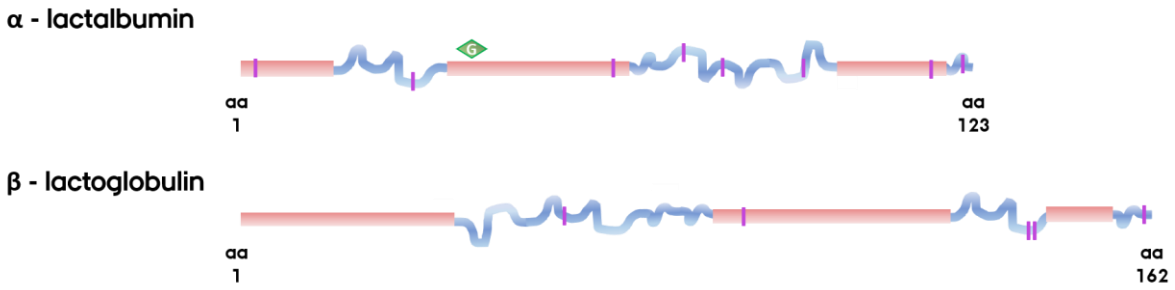


Figure 4. Post translational modifications of the major whey proteins based on reviews by Farrell et al. (2004) and Caroli et al. (2009). Blue stretches: hydrophilic regions, red stretches: hydrophobic regions. Purple lines indicate positions of Cys. G: glycosylation. Figure from Poulsen & Larsen (2022).

Table 3. Central structural features of the major milk proteins, based on reviews by Farrell et al. (2004), Caroli et al. (2009), Le et al. (2017).

Protein	α_{S1}-CN	α_{S2}-CN	β-CN	κ-CN	α-LA	β-LG
#Amino acids – mature protein	199	207	209	169	123	162
Signal peptide – size aa	15	15	15	21	19	16
#Genetic variants in *Bos*	9	4	12	14	3	11
Reference protein (file)	B-8P (P02662)[1]	A-11P (P02663)[1]	A^2-5P (P02666)[1]	A-1P (P02668)[1]	B (P00741)[1]	B (P02754)[1]
Size (Da)	23,615	25,226	23,983	19,037	14,178	18,277
Phosphorylation (P)	8-9P	10-14P	4-5P	0-3P	-	-
Glycosylation	No	No	No	Yes	Yes	No
Disulphide bridges (S-S)	-	2 (mainly dimer)	-	2 (mainly multimer)	4	2
Fraction in CN or whey	40% of CN	10% of CN	45% of CN	5% of CN	25% of WP	50% of WP

[1]UniProt accession number.

The relative content of major milk proteins, their isoforms and glycosylation- and major phosphorylation states are presented in Table 4 for Danish Holstein and Danish Jersey. The quantitative results are based on UV signal from individual peaks detected by liquid chromatography (LC)-based separation coupled with mass spectrometry (MS), LC/ESI-MS analysis relative per run, and protein forms identified by their masses, as indicated in Jensen et al. (2012b). Milk from Danish Holstein cows is mainly characterised by higher relative contents of β-CN, α-lactalbumin, β-lactoglobulin and higher fraction of glycosylated κ-CN (G κ-CN) to total κ-CN, whereas milk from Danish Jersey cows was mainly characterised by higher relative contents of κ-CN, α_{S2}-CN, and the less phosphorylated forms of α_{S1}-CN and α_{S2}-CN. These results are in line with those reported by Gustavsson et al. (2014a) using the same data, but where the protein profile was based on capillary zone electrophoresis. Univariate linear models including days in milking and parity as class effects showed variation in the detailed protein profile across and between lactations, and in particular changes in the degree of glycosylation of κ-CN were pronounced, but also changes in α_{S1}-CN 8P to total α_{S1}-CN and α_{S2}-CN 11P to α_{S2}-CN were observed over lactation for both breeds (Poulsen et al., 2016a).

Table 4. Descriptive statistics of relative protein composition and individual proteins content in Danish Holstein and Jersey cows. Adapted from Poulsen et al. (2016).

	Danish Holstein			Danish Jersey		
Trait[1]	Mean	Min – max	CV%	Mean	Min – max	CV%
Total α_{S1}-CN%	27.0[a]	14.8 - 32.8	9.6	27.4[b]	12.5 - 41.2	10.9
α_{S1}-CN 8P	21.0[a]	7.8 - 25.3	10.1	21.2[b]	9.7 - 30.4	11.2
α_{S1}-CN 9P	6.9[a]	3.1 - 10.8	18.5	6.2[b]	2.0 - 10.7	23.3
α_{S2}-CN%	4.9[a]	2.6 - 9.5	20.9	5.4[b]	2.4 - 9.9	24.7
α_{S2}-CN 11P	3.0[a]	1.4 - 5.2	22.2	3.6[b]	1.4 - 6.6	25.4
α_{S2}-CN 12P	1.9[a]	0.8 - 4.3	27.6	1.7[b]	0.7 - 3.7	30.2
β-CN%	36.0[a]	24.1 - 43.0	7.4	29.1[b]	15.1 - 39.0	14.1
Total κ-CN%	5.9[a]	3.5 - 8.4	16.1	7.0[b]	3.3 - 83.9	10.8
G κ-CN%	1.4[a]	0.5 - 3.5	33.9	1.4[a]	0.6 - 3.4	27.7
UG κ-CN%	4.5[a]	2.6 - 6.8	16.4	5.5[b]	1.3 - 7.7	11.9
α-lactalbumin%	3.1[a]	1.0 - 5.7	21.1	2.5[b]	0.5 - 4.2	21.8
β-lactoglobulin%	7.9[a]	3.1 - 13.9	19.0	6.4[b]	2.3 - 12.0	24.4
G κ-CN/total κ-CN	23.7[a]	9.3 - 45.7	27.4	20.4[b]	9.9 - 60.2	25.3
α_{S1}-CN 8P /total α_{S1}-CN	74.3[a]	52.5 - 87.0	5.2	77.4[b]	59.0 - 88.2	5.6
α_{S2}-CN 11P/total α_{S2}-CN	61.1[a]	45.9 - 80.8	10.1	67.5[b]	46.5 - 82.5	7.8
Protein (g/100 g milk)	3.44[a]	2.82 - 4.31	7.7	4.29[b]	2.65 - 5.45	7.7

[1]The individual proteins comprise the peaks identified as intact protein and isoforms marked in Jensen et al., (2012), i.e. α_{S1}-CN (comprise α_{S1}-CN 8P + 9P), α_{S2}-CN (comprise α_{S2}-CN 11P + 12P), β-CN (β-CN 5P), κ-CN (comprise κ-CN G + 1P). [a-b] Significant trait variation ($P < 0.05$) between Danish Holstein and Danish Jersey cows. G: Glycosylated, U: Un-glycosylated.

Frequency of genetic variants

The major milk proteins are found in different forms due to genetic polymorphism resulting in a number of amino acid changes. In the Milk Genomics project all animals were genotyped using a custom Taqman SNP genotyping assay targeting the known variants of the major proteins (Poulsen et al., 2013). By using LC-ESI/MS β-CN variant F was also for the first time recognized in low frequency in the Danish Holstein (Jensen et al., 2012b; Poulsen et al., 2016b). Genotype and allele frequencies of major genetic variants in Danish Holstein and Danish Jersey are shown in Table 5. For the *CSN1S1* gene (coding for α_{S1}-CN), variants B and C were identified. The most common *CSN1S1* genotype was BC in Danish Jersey and BB in Danish Holstein. For *CSN2* (coding for β-CN), six *CSN2* variants were resolved in Danish Holstein (A^1, A^2, A^3, B, I, and F), and 4 variants in Danish Jersey (lacking CSN2 A^3 and F). For *CSN2*, A^2A^2 was identified as the most common genotype in both Danish Holstein and Danish Jersey table 5. Finally, for *CSN3* (coding for κ-CN), three variants were present in Danish Holstein compared with Danish Jersey, where *CSN3* E was not detected. For *CSN3*, the *CSN3* BB genotype was dominating in Danish Jersey, and the AA genotype was dominating in Danish Holstein (Table 5) genomic organization of the CN loci on chromosome 6 is *CSN1S1-CSN2-CSN1S2-CSN3* (Threadgill and Womack, 1990). As the casein genes are closely linked, linkage disequilibrium, which describes the non-random association of the casein alleles is very likely. As expected, linkage disequilibrium was detected between all pairs of loci in Danish Jersey ($P < 0.001$); between *CSN1S1* and *CSN2* ($P < 0.001$), and *CSN2* and *CSN3* ($P < 0.001$) in Danish Holstein. In Danish Jersey, the common *CSN1S1* C appeared strongly associated with *CSN2* A^2 and *CSN3* B, as all *CSN1S1* CC homozygotes were also homozygotes for *CSN2* A^2A^2 and *CSN3* BB. Also, heterozygous *CSN1S1* BC always co-occurred with *CSN2* A^2 and *CSN3* B in Danish Jersey. This was not detected in Danish Holstein. Similarly, strong linkage disequilibrium was observed for *CSN2* A^1 and *CSN3* E, and between *CSN2* I and *CSN3* B.

Table 5. Genotype and allele frequencies of α_{S1}-CN (CSN1S1), β-CN (CSN2), κ-CN (CSN3) and β-lactoglobulin (β-LG) in Danish Holstein (DH) and Danish Jersey (DJ) cows. α_{S1}-CN and κ-CN frequencies adapted from Poulsen et al. (2013), β-CN frequencies adapted from Poulsen et al. (2016b). β-LG frequencies calculated based on LC-ESI/MS data.

	Genotype frequency			Variant frequency		
Protein	Genotype	DH	DJ	Variant	DH	DJ
α_{S1}-CN	BB	0.990	0.319	B	0.995	0.57
	BC	0.010	0.502	C	0.005	0.43
	CC	-	0.179			
β-CN	A^1A^1	0.062	0.007	A^1	0.254	0.081
	A^1A^2	0.330	0.094	A^2	0.621	0.629
	A^2A^2	0.376	0.415	A^3	0.004	-
	A^1A^3	0.002	-	B	0.044	0.22
	A^2A^3	0.007	-	I	0.069	0.07
	A^1I	0.033	0.012	F	0.008	-
	A^2I	0.080	0.088			
	II	0.007	0.002			
	BI	0.011	0.037			
	BA^1	0.018	0.041			
	BA^2	0.060	0.247			
	BB	0.000	0.058			
	A1F	0.002	-			
	A2F	0.013	-			
κ-CN	AA	0.480	0.039	A	0.696	0.206
	AB	0.348	0.333	B	0.240	0.794
	BB	0.050	0.627	E	0.064	-
	AE	0.086	-			
	BE	0.031	-			
	EE	0.005	-			
β-LG	AA	0.261	0.316	A	0.538	0.546
	AB	0.553	0.429	B	0.462	0.422
	BB	0.186	0.194	C	-	0.032
	BC	-	0.028			
	AC	-	0.032			
	CC	-	0.002			

Association of PTMs with protein genetic variants

It is interesting to ascertain to which extent the various protein genetic variants of the major milk proteins carry PTMs (here: glycosylations and phosphorylations), as it influences milk protein properties and micelle organisation. This was studied especially for κ-CN by various techniques in several studies, by different types of LC-based separation coupled with MS, LC-MS (LC/ESI-MS single quadrupole (Q) and LC/ESI-MS quadrupole Time-of-Flight (Q-TOF) MS/MS) or by gel-based separation combined with MS identification of excised spots (2-dimensional gel electrophoresis combined with Matrix-assisted Laser Ionization (MALDI) TOF MS/MS). By the gel-based approach, a sub-set of 24 samples from each of the two breeds, Danish Holstein and Danish Jersey, were selected based on rheological profiles by ReoRox, using the parameters rennet coagulation time (RCT) and curd firming rate (CFR) in relation to rennet coagulation properties, defining two coagulation groups with either good or poor coagulation abilities within breeds (Jensen et al., 2012b). By the gel-based study it was found that 95 % of the κ-CN molecules in a pooled milk sample were phosphorylated (1 or 2 P), and 36 % were glycosylated (identified with 1, 2, or 3 O-glycans) in Danish Holstein, and 96% and 34 % for Danish Jersey, i.e. almost the same shares between the two breeds (Jensen et al., 2012a). Even though small variations were seen between PTM forms of the genetic variants (A, B, E) of κ-CN, these were not significantly different by this gel-based approach. In an additional study by detailed LC-ESI/MS Q-TOF MS/MS using an even smaller subset of genotyped samples (12 from Danish Holstein and 17 from Danish Jersey) with distinct κ-CN genotypes, it was confirmed, as earlier indicated in the literature, that the share of glycosylated κ-CN/total κ-CN was higher for BB variant in Danish Holstein compared with both AA, AB, EE and AE genotypes (Jensen et al., 2015a).

The glycosylation degree representing the amount of glycosylated κ-CN forms relative to total κ-CN in relation to the different genetic κ-CN variants was finally studied including milk from all sampled Danish cows (456 Danish Holstein, 436 Danish Jersey, excluding all with somatic cell count > 500.000 cells/ml) using LC/ESI-MS. It was found (Figure 5) that κ-CN BB showed higher relative contents of both un-glycosylated κ-CN and glycosylated κ-CN compared with κ-CN AA, and κ-CN AB showed intermediate results in both breeds (Poulsen et al., 2016).

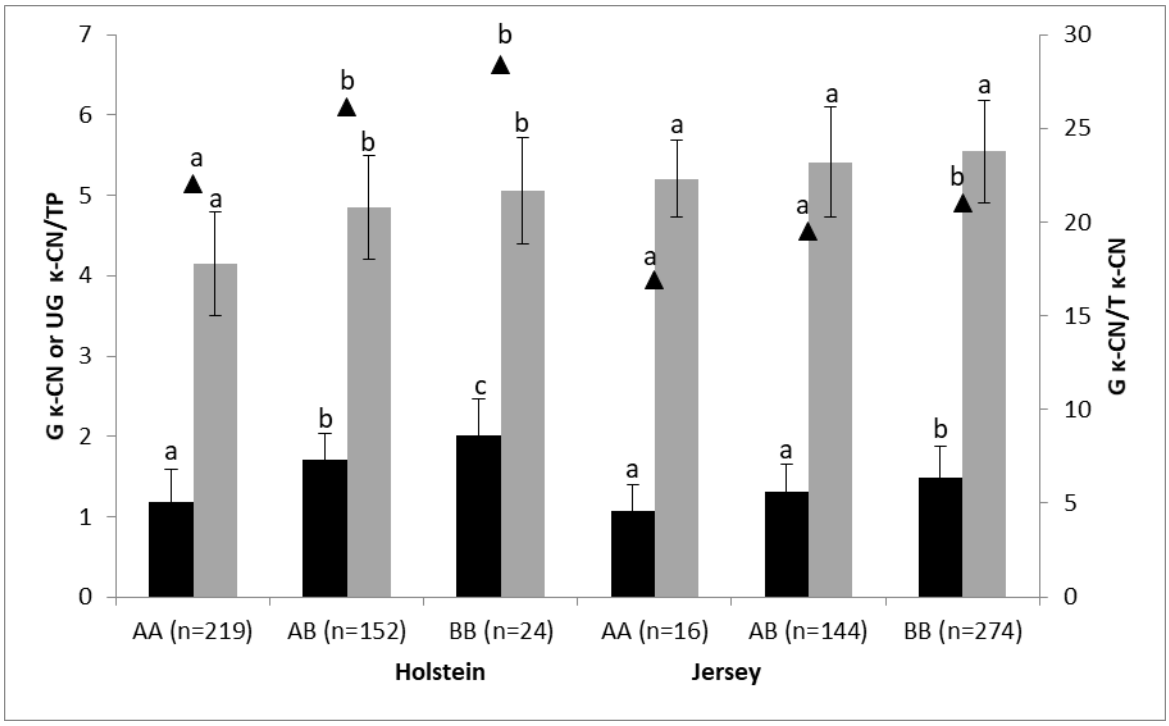


Figure 5. Content (%) of glycosylated κ-CN relative to total protein (G κ-CN/TP, black), un-glycosylated κ-CN relative to total protein (UG κ-CN/TP, grey), and glycosylation degree (G κ-CN/κ-CN, ▲) in milk from Danish Holstein and Danish Jersey cows. Letters indicate different contents between genotypes within breeds (P<0.05). Figure adapted from Poulsen et al. (2016).

Absolute quantification of α-lactalbumin, β-casein and osteopontin

As part of a study of variation in content and breeding potential, ingredient proteins with bioactive or functional properties were selected and their absolute concentrations in a number of individual cow's milk samples from Danish Holstein were determined. The determination of absolute concentrations of specific proteins (e.g. in mg/l) is in contrast to the usual relative distribution obtained by LC/ESI-MS and require the use of specific and pure standards, which can be challenging to obtain. The absolute levels of α-lactalbumin, β-CN and osteopontin were determined in 663 individual cow's milk samples from Danish Holstein, including samples from both the Milk Genomics sampling and from a subsequent study on Holstein milk. Osteopontin was determined by a specifically developed sandwich ELISA. α-lactalbumin and β-CN were determined by specifically developed Multiple Reaction Monitoring (MRM) run on LC/ESI-MS triple Q equipment. The developed MRM method was based on the quantification of specific peptides from the proteins. These peptides were generated by reproducible enzymatic cleavage by trypsin, representing unique parts of the protein sequence. The level of osteopontin varied from 0.4 to 68 mg/l, with an average of 25 mg/l. Osteopontin concentration has earlier been reported to around 18 mg/l, but has never before been reported in such a large number of individual cow's milk samples. An effect of parity on the level of osteopontin (OPN) was observed, with decreasing levels at higher parity (Christensen et al., 2021). The level of α-

lactalbumin determined by MRM varied from 0.5 to 1.9 g/l, with an average of 1.1 g/l. Levels of β-CN varied from 7.5 to 23.7 m/l, with an average of 14.9 g/l (Table 6) (Le et al., 2020).

Table 6. Absolute levels of OPN, β-CN and α-lactalbumin in individual cow's milk samples as determined by MRM. Adapted from Le et al. (2020); Christensen et al. (2021) and Poulsen et al. (2018).

	Danish Holstein		
Trait[1]	Mean	Min – max	CV%
Osteopontin (mg/L)	25.03	0.40-67.80	41.80
β-CN (g/L)	14.92	7.53-23.74	16.65
α-lactalbumin (g/L)	1.06	0.54-1.88	21.16

1.3 Vitamins

Undoubtedly, bovine milk is one of the best sources for several vitamins and minerals in human nutrition, including riboflavin (vitamin B2) and cobalamin (vitamin B12). Riboflavin belongs to the essential water-soluble vitamins in milk according to Haug et al. (2007), and plays a key role in numerous metabolic pathways and redox reactions through the biologically active coenzymes, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) (Powers, 2003). In particular, for elderly people and adolescents, low intake of riboflavin-containing foods can result in riboflavin deficiencies and, in Western diets, milk and dairy products account for approximately 51% of the intake in preschool children (Powers, 2003). Milk is known to contain other important vitamins as well, e.g. vitamin D, but these were not part of the measurements in the present study.

Despite the acknowledged value of milk and dairy products as riboflavin sources (Sunaric et al., 2012), very few studies have documented the drivers for riboflavin variation in milk within and across bovine breeds. The primary origin of the water soluble riboflavin and other B vitamins is through microbial biosynthesis in the rumen (Schwab et al., 2006). Documented effects of feed and breed (Shingfield et al., 2005; Poulsen et al., 2015b) are related to the rumen environment and the microbial processes responsible for the riboflavin synthesis. Poulsen, et al. (2015b) found substantial interbreed differences in milk riboflavin content. Milk from Danish Jersey cows contained significantly higher levels of riboflavin (1.93 mg/L milk) than milk from Danish Holstein cows (1.40 mg/L milk, Table 7). These concentrations are within the range reported in the literature (Lindmark-Månsson et al., 2003; Haug et al., 2007), and the difference between breeds were quite similar to what has previously been reported (Hand and Sharp, 1939; Theophilus and Stamberg, 1945).

Of the lipid-soluble vitamins in milk, α-tocopherol, which is the major type of vitamin E in bovine milk, also serves as an important antioxidant acting as a radical scavenger (Lindmark-Månsson and Åkesson, 2000). The level of antioxidants in milk plays an important role in relation to the oxidative stability, preventing oxidation of unsaturated fatty acids, and has been shown to be affected by different feed components. We found that the content of α-tocopherol was significantly higher in Danish Jersey compared to Danish Holstein (Table 7) (Poulsen et al., 2012). Higher levels of α-

tocopherol have been related to high pasture proportions in the feed (Havemose et al., 2004). Thereby, a higher level of antioxidants are correlated with higher levels of unsaturated fatty acids (Slots et al., 2009), probably due to higher levels of those FAs and vitamins in pasture in general (Havemose et al., 2004).

Table 7. Descriptive statistics of riboflavin and α-tocopherol contents in milk from Danish Holstein and Danish Jersey. Adapted from Poulsen et al., (2012, 2015a).

	Danish Holstein			Danish Jersey		
Trait[1]	Mean	Min – max	CV%	Mean	Min - max	CV%
Riboflavin mg/L[1]	1.40[a]	0.73-2.83	22.96	1.93[b]	1.02-2.84	15.72
α-Tocopherol µg/g[2]	20.30[a]	9.43-39.83	26.44	22.80[b]	9.30-53.42	26.94

[1]Riboflavin was determined by HPLC, essentially as described in Poulsen et al. (2015b) and presented as mg/L milk in 428 Danish Holstein and 395 Danish Jersey milk samples.
[2]α-tocopherol was determined by HPLC as outlined by Havemose et al. (2004) and presented as µg α-tocopherol per g of fat in 456 Danish Holstein and 435 Danish Jersey milk samples.

1.4 Minerals

Bovine milk provides important minerals, essential for both human nutrition and dairy product quality. The mineral fraction constitutes a minor fraction of the milk solids (approximately 7.1 to 7.4 g/L), and comprises cations, including e.g. calcium (Ca), magnesium (Mg), sodium (Na) and potassium (K), and anions, including e.g. phosphorus (P) and chloride (Cl) (Lindmark-Månsson et al., 2003; Hermansen et al., 2005). Minerals contribute to important physiological processes. It has been shown that Ca and P play a role in bone metabolism, Se and Zn play a role in the immune system, while Ca, K and Mg are involved in maintaining blood pressure (Cashman, 2006; Haug et al., 2007; Overton and Yasui, 2014). Furthermore, the mineral composition is important for the technological properties of milk, as minerals are involved in the structure and stability of casein micelles (micellar bound) and thereby e.g. the coagulation properties of the milk (Holt, 1992; Fox, 2009).

As mentioned, the minerals in milk exist in a dynamic equilibrium between a soluble serum phase and the colloidal micellar phase. Total Ca in milk is around 1.2 g/L, corresponding to approx. 30 mM. Of this, 0.8 g/L (approx. 20 mM) is in the micellar phase, and about 0.4 g/L (10 mM) is in the serum phase. The serum phase is divided into the ionic phase and the part bound to other molecules, like organic acids (mainly citrate, phosphate), amino acids, and serum (whey) proteins. This means that about 65% of the Ca in bovine milk is bound to the caseins. Of the soluble or serum phase Ca pool, approximately 2 mM is ionic, as Ca^{2+}. All these phases are in equilibrium in the milk and are influenced by many factors. Free divalent cations, especially Ca^{2+} in milk serum, significantly influence the surrounding environment of the negatively charged casein micelles (Tsioulpas et al., 2007) and thereby the coagulation properties of the milk through molecular interactions, as e.g. important in the second phase of rennet induced milk coagulation.

We have reported concentrations of ten different elements (Ca, Cu, Fe, K, Mg, Mn, Na, P, Se, and Zn (Table 8; Buitenhuis et al., 2015) in relation to Danish dairy breeds. Milk from Danish Holstein has a lower mineral content (Ca, Cu, Fe, Mg, Mn, Na, P, Se, and Zn) compared to Danish Jersey, except for K, which is higher in the Danish Holstein (1469.8 ppm±115.0 Danish Holstein vs. 1319.0 ppm ±104.9 Danish Jersey); however, the CV% was comparable between the two breeds (Table 8). Gaucheron (2005) stated that milk mineral content is relatively constant; however, the present study shows that there is substantial variation with regards to the different minerals in bovine milk. This is in line with previous results showing considerable variation in milk mineral content from Swedish and Danish herds especially due to season, but also to breed (Lindmark-Månsson et al., 2003; Hermansen et al., 2005). Results by van Hulzen et al. (2009) also showed considerable variation for mineral content in milk of Dutch Holstein-Friesians caused by genetic and/or environmental and nutritional variation.

Table 8. Descriptive statistics of micro and macro elements in Danish Holstein and Danish Jersey milk (mg/l). Contents modified according to Buitenhuis et al. (2015).

	Danish Holstein			Danish Jersey		
Trait[1]	Mean	Min – max	CV%	Mean	Min - max	CV%
Ca	1214[a]	938-1704	10.08	1465[b]	1047-1925	10.06
K	1470[a]	1170-1819	7.81	1319[b]	911-1592	7.96
Na	349[a]	250-783	21.08	389[b]	237-1016	25.96
P	725[a]	533-954	10.68	880[b]	623-1117	10.57
Mg	108[a]	85-143	9.87	124[b]	91-167	10.37
Cu	0.03[a]	0.01-0.11	45.38	0.05[b]	0.01-0.18	46.60
Fe	0.17[a]	0.10-0.52	22.80	0.19[b]	0.11-0.83	27.30
Mn	0.02[a]	0.01-0.04	27.99	0.03[b]	0.01-0.07	29.61
Se	0.007[a]	0.004-0.014	28.60	0.011[b]	0.006-0.019	21.31
Zn	3.39[a]	1.70-5.52	18.58	4.73[b]	2.50-7.13	16.91

[1]Minerals were extracted from skimmed milk by acid sonication and identified using inductively coupled plasma mass spectrometry (ICP-MS) as described by Cava-Montesinos et al. (2005). Levels are presented in ppm for 314 Danish Holstein and 316 Danish Jersey. Different superscript letters within a row indicate significant ($p<0.05$) differences between means.

Phenotypic correlations between the mineral and overall milk composition show similarities in milk from both Danish Holstein and Danish Jersey (Table 9). Especially P, Ca and Mg were positively inter-correlated, and displayed a further strong correlation to protein content. This is in line with Bijl et al. (2013), and is likely to contribute to the higher contents of these minerals in Danish Jersey milk. This relationship is due to the association of these minerals with the casein micelles, and is also known to be of utmost importance for casein micelle stability (Gaucheron, 2005; Deeth and Lewis, 2015). Thereby their concentrations also affect the technological properties of milk, and lower Ca levels (and to some extent lower levels of P and Mg) have been associated with poor or non-coagulating milk (Hallén et al. 2010; Jensen et al. 2012a; Jensen et al. 2012b). The association between these mineral fractions and milk coagulation properties will be discussed in further details below.

Milk contents of Cu, Fe, Mn, Se and Zn further tended to be higher in Danish Jersey as compared to Danish Holstein, which is in accordance with Hermansen et al. (2005). The Cu concentration in milk is known to vary both between individual cows, and with diet and level of mineral supplementation (Dunkley et al., 1968). Previously it has been shown that the Cu concentration plays an important role in the spontaneous development of oxidative off-flavour of the milk (Juhlin et al., 2010, 2012). The mineral contents reported here were based on skimmed milk, which could have affected the reported levels, as small amounts may be associated with the milk fat fraction. For instance, phosphor from phospholipids in the milk fat globule membrane would not have been included, which will have an effect on the milk P level as compared to earlier studies on full milk (Lindmark-Månsson et al., 2003; Hermansen et al., 2005). The only larger difference in the correlation association within breeds was a negative correlation between lactose and K (-0.22±0.06) in DH, while these components have a positive correlation (0.17±0.06) in Danish Jersey (Table 9). It is not known what drives this variation.

Table 9. Phenotypic correlation of between milk production traits and the mineral content of the milk. Above the diagonal the phenotypic correlation for Danish Holstein. Below the diagonal the phenotypic correlation for Danish Jersey. Correlation coefficients > 0.4 highlighted in bold. Adapted from Buitenhuis et al. (2015).

Trait	Fat	Protein	Casein	Lactose	Ca	Cu	Fe	K	Mg	Mn	Na	P	Se	Zn
Fat		**0.43**	**0.43**	-0.25	0.37	0.37	0.32	-0.02	0.19	0.09	-0.10	0.32	0.04	0.13
Protein	**0.46**		**0.95**	-0.11	**0.44**	0.35	0.29	-0.01	0.33	0.37	0.02	**0.51**	0.12	0.38
Casein	**0.41**	**0.93**		-0.002	**0.49**	0.38	0.28	-0.09	0.33	0.35	-0.06	**0.46**	0.14	0.37
Lactose	**-0.41**	-0.13	0.03		-0.004	0.02	-0.14	-0.22	-0.19	-0.08	-0.39	0.10	-0.1	0.05
Ca	**0.43**	**0.55**	**0.63**	0.006		0.25	0.26	0.04	**0.45**	0.19	-0.07	**0.55**	0.09	0.28
Cu	0.24	0.21	0.23	0.01	0.28		0.15	0.05	-0.04	0.12	-0.15	0.27	0.17	0.17
Fe	0.33	0.24	0.17	-0.26	0.18	0.07		-0.07	0.15	0.33	0.18	0.12	0.26	0.17
K	-0.07	0.05	0.08	0.17	0.33	0.06	-0.02		0.06	-0.07	-0.38	0.33	-0.06	0.08
Mg	0.36	**0.48**	**0.45**	-0.22	**0.52**	-0.06	0.14	0.25		0.07	0.14	0.38	0.11	0.12
Mn	0.18	0.39	0.38	-0.02	0.23	0.12	0.13	-0.02	0.18		0.23	0.26	0.23	0.31
Na	-0.03	-0.003	-0.24	**-0.56**	-0.21	-0.12	0.14	**-0.45**	-0.001	0.11		-0.16	0.19	-0.04
P	0.26	**0.47**	0.39	0.19	**0.59**	0.13	0.09	**0.47**	**0.48**	0.28	-0.12		0.038	0.37
Se	0.16	0.1	0.03	-0.2	0.16	0.11	0.16	0.01	0.21	0.08	0.21	0.12		0.13
Zn	0.26	**0.47**	**0.45**	0.01	**0.41**	0.2	0.13	0.07	0.36	0.44	-0.02	**0.44**	0.15	

1.5 Oligosaccharides

Free oligosaccharides (OS) are bioactive molecules present in human milk that provide numerous health benefits to developing infants. This includes stimulating growth of selected beneficial bacteria in the gut, participating in development of the brain and exerting anti-pathogenic activity by preventing pathogen binding to intestinal epithelial cells (Pacheco et al., 2015; Jacobi et al., 2016). Despite some differences in abundance, structural complexity, and diversity between human and bovine OS, bovine milk contains several OS structures in common with human milk (Aldredge et al., 2013; Charbonneau et al., 2016; Cohen et al., 2017; Bell et al., 2018; Kirmiz et al., 2018). Given the vast amount of whey originating from cheese production, recovery and up-concentration of bovine milk OS from dairy streams could be a valuable source of OS for use as bioactive ingredients, especially for the purposes of enhancing the functionalities of infant formula and developing value-added ingredients for nutraceutical applications (Barile et al., 2009; Barile and Rastall, 2013; Mehra et al., 2014).

Bovine milk OS are generally smaller in size than those of human milk, with less complex structures and fewer isomers for each composition (Tao et al., 2008). Milk OS are synthesised from glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), fucose (Fuc) (Pacheco et al., 2015), N-acetylneuraminic acid (NeuAc) and N-glycolylneuraminic acid (NeuGc) (Boehm and Stahl, 2007), likely by the action of specific glycosyltransferases (Wickramasinghe et al., 2011Poulsen et al., 2019). Initially, a small set of milk samples from Danish Holstein and Danish Jersey cattle were analysed and differences in OS abundances were found between breeds (Sundekilde et al., 2012). OS in milk from Danish Jersey cows contained higher relative amounts of both acidic (sialic acid containing OS) and the more complex neutral fucosylated OS, whereas milk from Danish Holstein had a higher abundance of smaller and simpler neutral OS (Sundekilde et al., 2012). That particular study also revealed that bovine milk contains several larger fucosylated structures (containing up to 10 monosaccharide units) and not just simple OS as previously thought. To quantify OS in a larger data set isobaric tags for an optimised MS-based OS quantification method was used (Robinson et al., 2018), which has enabled relative OS quantification in more than 600 milk samples from Danish Holstein and Danish Jersey, Table 10 (Poulsen et al., 2019; Robinson et al., 2019). The results confirmed that Danish Jersey milk contains higher amounts of most bovine OS, including more fucosylated OS. In both breeds, variation in OS abundance was strongly affected by parity (Robinson et al., 2019).

Table 10. Mean, standard deviation (SD) and coefficient of variation (CV%) of relative oligosaccharide abundances in milk from Danish Holstein and Danish Jersey. Adapted from Poulsen et al. (2019).

	Danish Holstein			Danish Jersey		
Trait[1,2]	Mean	SD	CV%	Mean	SD	CV%
2_0_0_1_0 (3'-SL)	0.788	0.280	35.5	1.300	0.478	36.8
2_0_0_1_0 (6'-SL)	0.716	0.326	45.6	1.351	0.539	39.9
2_0_0_2_0	1.046	0.410	39.2	1.681	0.735	43.7
2_1_0_0_0 isomer 1	1.406	1.211	86.1	1.481	1.414	95.5
2_1_0_0_0 isomer 2	1.320	0.490	37.1	1.300	0.526	40.5
3_1_0_0_0 (LNT)	0.938	0.302	32.2	1.686	1.133	67.2
3_1_0_0_0 Isomer 2	0.527	0.295	56.1	1.134	0.403	35.6
3_2_0_0_0	0.787	0.612	77.8	1.835	1.407	76.6
3_6_1_0_0	0.577	0.208	36.1	1.267	1.065	84.1
4_1_0_0_0	0.932	0.590	63.3	2.198	1.193	54.2
4_2_0_0_0 (LNH)	0.495	0.195	39.4	1.609	1.408	87.5
4_4_1_0_0	0.706	0.304	43.1	1.294	0.649	50.1
4_5_1_0_0	0.775	0.354	45.7	1.664	1.917	115.2
5_4_0_0_0	0.705	0.426	60.5	0.383	0.261	68.2
5_4_1_0_0	0.845	0.319	37.7	1.215	0.722	59.4

3'-SL = 3'-sialyllactose, 6'-SL = 6'-sialyllactose, LNT = Lacto-N-tetraose, LNH = Lacto-N-hexaose.
[1]Oligosaccharides are represented by their monosaccharide compositions, denoted as Hex_HexNAc_Fuc_NeuAc_NeuGc.
[2]OS abundance values are expressed as the mass spectral intensity of the isobaric label reporter ions relative to that of a spiked internal standard of the same parent mass. OS collected from a standardized preparation of bovine milk OS powder were used as the internal standard mixture and spiked into each multiplexed set.

1.6 Metabolites

In total, 38 metabolites were identified in milk (Table 11). Each metabolite was relatively quantified by integration of NMR resonance signals. Lactose, citrate and urea were measured both by NMR and by infrared spectroscopy (Milkoscan). The metabolites with the highest CVs were cis-aconitate (Holstein: 269.95%; Jersey: 359.07%), whereas galactose had the lowest CV (Holstein: 9.00%; Jersey: 8.29%). For the 2009 and 2010 sampling, calibration samples for urea and citrate were not provided for the Milkoscan instrument, and the accuracy of their concentrations is therefore not validated, but both traits are highly correlated with the NMR traits.

Table 11. Relative quantification (mean relative to total identified), standard deviation (SD) and coefficient of variation (CV%) for metabolite levels in Danish Holstein and Danish Jersey milk. Results are also presented, but on a smaller subset, in Buitenhuis et al. (2013).

	Danish Holstein			Danish Jersey		
Trait	Mean	SD	CV%	Mean	SD	CV%
2-oxoglutarate	1.12	0.28	25.13	0.87	0.17	19.88
3-hydroxybutyrate	1.03	1.35	131.56	0.97	0.53	54.31
Acetate	1.02	1.88	185.11	0.99	2.34	236.57
Acetone	1.14	0.56	48.90	0.86	0.38	44.47
Alanine	1.14	0.32	28.59	0.86	0.27	31.92
Betaine	1.18	0.60	51.45	0.82	0.49	60.22
Butyrate	1.15	0.86	75.01	0.84	0.70	83.69
Caprylate	1.04	0.42	40.70	0.96	0.35	36.14
Carnitine	1.15	0.42	36.50	0.84	0.33	39.72
Choline	0.68	0.31	45.91	1.34	0.38	28.12
cis-Aconitate	1.51	4.08	269.95	0.45	1.63	359.07
Citrate	0.94	0.16	17.29	1.06	0.14	13.18
Creatinine	1.03	0.39	38.28	0.97	0.34	35.06
Fucose	0.75	0.53	71.17	1.26	0.66	52.42
Fumarate	1.02	0.40	39.67	0.98	0.28	28.34
Galactose	0.99	0.09	9.00	1.01	0.08	8.29
Galactose-1-phosphate	0.95	0.82	86.57	1.05	0.85	80.59
Glucose	0.91	1.09	119.42	1.08	1.31	120.65
Glucose-1-phosphate	1.01	1.45	143.64	0.99	1.43	144.84
Glutamate	1.25	0.50	39.75	0.74	0.30	39.72
Glycerophosphocholine	1.01	0.17	16.77	0.98	0.15	15.12
Hippurate	1.11	0.32	28.47	0.88	0.25	28.70
Isobutyrate	0.97	0.59	60.95	1.04	0.55	52.79
Isoleucine	1.03	0.64	62.76	0.97	0.75	77.52
Lactate	0.98	1.18	119.98	1.02	1.90	186.50
Lactose	1.02	0.13	13.08	0.98	0.14	14.68
Leucine	1.00	0.60	60.71	1.01	0.81	80.77
Malonate	0.94	0.23	24.21	1.06	0.45	42.69
Methionine	1.34	0.82	61.29	0.65	0.53	81.59
N-acetyl-carbohydrates	1.06	0.42	39.89	0.94	0.34	36.83
O-acetylcholine	1.04	0.26	25.20	0.96	0.26	26.86
O-phosphocholine	0.95	1.13	119.07	1.06	1.63	153.77
Orotate	1.14	0.43	37.67	0.85	0.23	27.36
Pantothenate	0.99	0.27	27.53	1.01	0.28	27.98
Tryptophan	0.97	0.64	66.24	1.03	0.64	62.51
Urea	0.93	0.22	23.37	1.07	0.22	20.91
Uridine	1.07	0.55	51.83	0.93	0.46	50.02
Valine	1.04	0.38	36.64	0.96	0.49	51.15

Lactose

The reducing disaccharide lactose is the most abundant carbohydrate in milk and consists of galactose and glucose linked by a β1-4 glycosidic bond (β-D-galactopyranosyl-1,4-D-glucose). Milk is the only known source of lactose, and its concentration in milk ranges from 0 in California sea lions to 10% in Green monkey. On average human milk contains 7.0%, whilst bovine milk contains 4.8% lactose (Fox and McSweeney, 1998). Milk is isotonic with blood, and lactose is responsible for 50% of the osmotic pressure of milk. Thus, milk with a low level of lactose has a high level of inorganic salts or other compounds in order to maintain the osmotic pressure (Fox and McSweeney, 1998).

Citrate

Citrate, as citric acid, is around 9.2 mM (1.8 g/L), of this 89% is diffusible (soluble) and 11% colloidal (Gaucheron, 2005). Natural levels have been reported to vary dependent on factors related to production, feeding, lactation stage, as well as measurement methods. This diffusible whey-based citrate fraction can influence calcium distribution by complexing with ionic calcium and thereby influence calcium balance. Citrate was measured by both Milkoscan e.g. by Jensen et al. (2012b) and NMR (Sundekilde et al., 2011). Milkoscan data is reported in Table 16 and citrate was found to be in the range of 1.1-2.9 g/L. Citric acid is involved in the fatty acid metabolism. The conjugated base of citric acid is citrate, which is important in the fat metabolism. Citrate is transported to the cytoplasm, where it is converted to acetyl CoA. Acetyl CoA is then converted into malonyl CoA by the acetyl CoA carboxylase.

Urea

Differences in milk urea levels are associated with the content of dietary protein in the feed and digestion in the rumen, where excess ammonia from protein digestion will be transferred to the blood stream and converted to urea in the liver. A positive correlation therefore exists between dietary crude protein content and milk urea content, and milk urea nitrogen can be used as a diagnostic indicator of protein feeding (Nousiainen et al., 2004). Milk urea nitrogen (MUN) can be measured by infrared spectroscopy using Milkoscan, and variation in MUN from 3.8 to 27.0 mg/dL has been reported (Nousiainen et al., 2004). From our in-house Milkoscan, urea was not measured as MUN, but as milk urea in mg/L. Results from urea measurements in present study on Danish dairy cows milk can be seen in Table 12.

2. Effect of elevated somatic cell count on milk composition

Somatic cell count (SCC) is associated with changes in milk composition, including changes in proteins, lipids, and milk metabolites. SCC is normally used as an indicator of mastitis infection. In Denmark, SCC is used as a general milk quality indicator, and the price of bulk milk with more than 400,000 somatic cells/mL is penalised by 10% in the payment system (Danish Dairy Board, 2020). In this project, milk was collected from healthy cows. When no sign of clinical mastitis or visual changes in the milk appearance are evident, milk from cows with elevated SCCs would normally be transferred to the farm bulk tank, and there could affect the quality of the tank milk (Le Maréchal et al., 2011). In most of our studies linked to the Milk Genomics publications reported here, milk with high SCC (>500,000 cells/mL) were excluded prior to analysis. However, in order to explore the effect SCC < 500,000 cells/mL on milk composition and quality, milk metabolites and milk compositional traits were compared in milk samples with high and low SCC (Sundekilde et al., 2013b; Poulsen et al., 2015c) from our dataset.

In Sundekilde et al. (2013b), NMR-based metabolomics was used to profile milk metabolites for differences related to SCC. Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA) on a smaller subset (n=70), representing high (SCC > 7.2×10^5 cells/mL) and low (SCC < 1.4×10^4 cells/mL) milk SCC, and partial leas squares (PLS) regression analysis on all sampled Danish Holstein and Danish Jersey cows pinpointed specific NMR spectral regions that differed according to SCC. Relative quantification of the identified metabolites revealed that lactate, butyrate, isoleucine, acetate, and β-hydroxybutyrate were increased, whereas lactose, hippurate and fumarate were decreased in milk with high levels of somatic cells (Sundekilde et al., 2013b).

In Poulsen et al. (2015c) milk samples with higher (>500,000 cells/mL) and lower (<500,000 cells/mL) SCC were compared within Danish Holstein and Danish Jersey cows (Table 12). Here, the significant change in lactose were confirmed, along with changes in conductivity and pH, which are indicative of subclinical mastitis. Lactose is the most important osmotic regulator in milk, and is very constant in most milk samples (Shennan and Peaker, 2000). At high SCC or in late lactation the mammary cell membrane is partly deteriorated and blood constituents and ions can flow into the milk (Bannerman, 2009), and in order to keep the osmotic pressure constant, lactose is decreased accordingly (Norberg, 2005). Short chain fatty acids (SCFA), including acetate, butyrate, propionate and lactate are end products of bacterial metabolism. Butyrate is the most simple of the SCFA in milk. Acetate, butyrate, and lactate have previously been shown to be increased in high SCC milk (Davis et al., 2004; Hettinga et al., 2008, 2009). Interestingly, a linear correlation between relative lactate concentration and SCC was observed in the present study. Klein et al. (2010) were unable to establish a correlation between lactate and SCC based on NMR data, however the study included a limited number of cows (Klein et

al., 2010). The association of isoleucine, hippurate, fumarate and β-hydroxybutyrate (BHBA) to SCC is less clear (Sundekilde et al., 2013b). In Poulsen et al. (2015c), cows with high SCC had a tendency toward longer rennet coagulation time (RCT) and lower curd firming rate (CFR), regardless of breed. The slightly impaired milk coagulation properties (MCP) of high SCC milk can be related to increased proteolytic degradation of caseins, mainly due to plasmin (Le Maréchal et al., 2011). Wedholm et al. (2008) showed that plasmin was primarily responsible for peptides in low SCC milk samples, whereas cathepsins and elastases played more prominent proteolytic roles in milk samples from cows with acute clinical mastitis (Wedholm et al., 2008). Therefore, the increased protein content observed in Danish Holstein is believed to reflect an increase in the whey protein levels, which is commonly reported in mastitic milk due to influx of blood proteins (Le Maréchal et al., 2011). Our data indicate, that milk from cows with no clinical signs of mastitis can be significantly different anyhow, and may cause economic losses for dairies. In addition to milk composition, SCC was also related to parity, with higher parity affecting the susceptibilities of cows to mastitis-causing pathogens.

Table 12. Comparison of milk with high (>500,000 cells/mL) and low (<500,000 cells/mL) somatic cell count (SCC) from Danish Holstein and Danish Jersey cows. Adapted from Poulsen et al. (2016).

	Danish Holstein			Danish Jersey		
	Low N = 398	High N = 35	Sign.	Low N = 409	High N = 25	Sign.
SCC	109 ± 108	1255 ± 1084	-	113 ± 108	1421 ± 1690	-
Parity	1.71 ± 0.76	2.11 ± 0.76	**	1.7 ± 0.77	2.2 ± 0.82	**
DIM	179 ± 21	184 ± 23	NS	186 ± 23	182 ± 22	NS
Yield (kg)*	14.75 ± 3.81	12.64 ± 3.42	**	10.16 ± 2.56	9.33 ± 3.50	NS
RCT (seconds)	1013 ± 202	1040 ± 203	NS	944 ± 141	1051 ± 229	**
CFR (Pa/min)	8.97 ± 4.18	8.59 ± 4.22	NS	21.65 ± 6.65	18.60 ± 6.36	NS
Protein %	3.43 ± 0.25	3.60 ± 0.32	**	4.31 ± 0.32	4.26 ± 0.27	NS
Fat %	4.00 ± 0.78	4.30 ± 1.05	*	5.99 ± 0.86	5.88 ± 0.82	NS
Lactose %	4.79 ± 0.13	4.66 ± 0.15	***	4.63 ± 0.14	4.46 ± 0.22	***
Conductivity (mS/cm)	5.82 ± 0.41	6.08 ± 0.40	***	5.43 ± 0.40	5.73 ± 0.62	**
Urea (mg/L)	210.2 ± 61.6	213.3 ± 62.9	NS	272.8 ± 59.7	284 ± 66.85	NS
Citric acid %	0.172 ± 0.03	0.165 ± 0.03	NS	0.182 ± 0.01	0.180 ± 0.02	NS
pH	6.68 ± 0.07	6.73 ± 0.07	***	6.66 ± 0.06	6.70 ± 0.11	*

*From morning milking

3. Milk Coagulation properties

The inclusion of coagulation properties as a central parameter in the Danish Milk Genomics project was performed on the shoulders of initial studies from mid-2000 and onwards, where we initially observed a surprisingly high number of non-coagulating milk among Swedish Red cows according to expected (Wedholm et al., 2006), as well as a significant amount of poorly coagulating milk in Danish Holstein and Danish Red, as well as a few non coagulating (Frederiksen et al., 2011b). During this period, there was an increasing interest in observations of non-coagulating milk in various studies, including Finnish Ayrshires and Swedish Red (Ikonen et al., 2004; Wedholm et al., 2006). This formed the background for the research aiming at deciphering genetic influence and milk compositional factors behind the non- and poorly coagulating milk properties. At this stage, it was not known, whether the impaired coagulation properties were linked to the first (enzymatic cleavage) or second (aggregation) phase of rennet induced milk coagulation. The coagulation problems could potentially also be linked to e.g. SCC and proteolysis or even presence of naturally occurring inhibitors of chymosin, like α_2-macroglobulin. Furthermore, it was not clear, whether the cows reverted between non- and good coagulation over time, and thereby also the genetic contribution to the trait variance was not evident. One further consideration was the potential contribution of the - at that time- newly found κ-CN E variant, which was associated with observed impaired coagulation properties in some cows. Findings of these initial studies have been published earlier (Wedholm et al., 2006; Frederiksen et al., 2011b), and have to some extent been included here.

Rennet-induced milk coagulation is important, as it is central in the process of cheese-making and is correlated with cheese yield and overall cheese quality (Ikonen et al., 1999; Wedholm et al., 2006), as well as in relation to process control. For a number of breeds a high frequency of non- and poor coagulating milk has been reported, which has been related to variation in milk compositional traits, health status, parity as well as genetic background. The milk coagulation properties were here measured by free oscillation and reported by two coagulation traits using ReoRox (Figure 6): Rennet Coagulation Time (RCT) and Curd Firming Rate (CFR). Rennet Coagulation Time was defined as the time from chymosin addition to the time, when the phase angle was 45° (θ = 45°). Curd Firming Rate was calculated from consecutive points of the linear part of the gelation profile $[\Delta G'/\Delta t]_{lin}$, thereby describing the storage modulus G' over time in minutes (see Frederiksen et al., 2011b). These two parameters both represent the *second* phase of rennet-induced milk coagulation. To minimise the variation, the coagulation experiments were made using Chy-Max Ultra (Chr. Hansen, Hørsholm, Denmark), which contains pure recombinant bovine chymosin, and furthermore all skim milk samples were adjusted to pH 6.5 ± 0.02 prior to chymosin addition. By pH adjustment, the contribution from variations in original milk pH to especially the first phase (enzymatic cleavage), of rennet-induced milk coagulation, is minimised. The final concentration of chymosin was 0.04 IMCU per mL milk in the sample cup.

Each milk sample was measured in technical duplicates, and the coagulation properties for individual milk samples were described by RCT and CFR. Maximum gel strength was not applied as a trait, because the gelation profile for several samples did not achieve maximum gel strength within the measuring period. Samples that did not coagulate within 1 h after chymosin addition were considered non-coagulating (Figure 6A and B; NC), as they did not exhibit a recordable RCT and had a CFR of 0. Good coagulating (GC) milk samples had a short RCT and high CFR compared to poor coagulating (PC) milk samples, which had longer RCT and lower CFR (Fig. 6B).

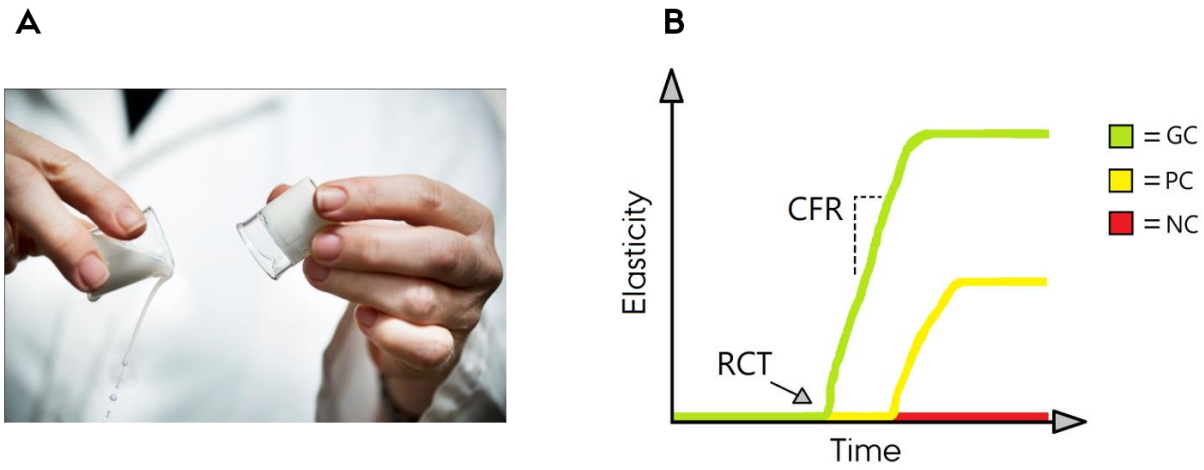
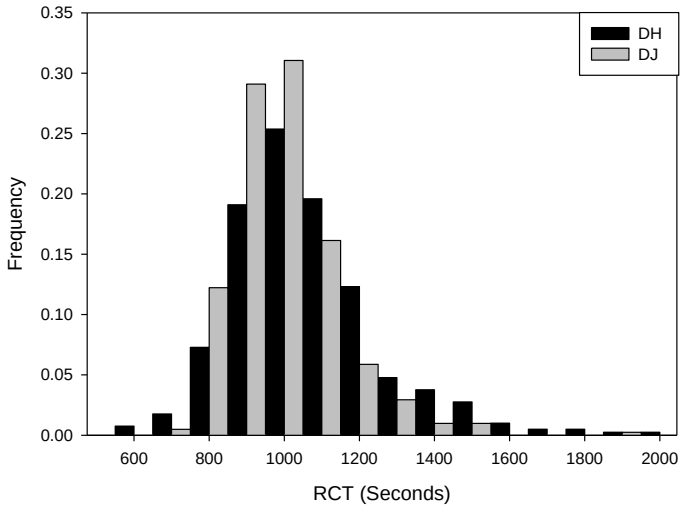


Figure 6. (A) Non-coagulating (NC) and good coagulating (GC) milk. (B) Representative gelation curves for NC, poor coagulating (PC) and GC milk, respectively. Onset of rennet coagulation time (RCT) and curd firming rate (CFR) are outlined. The curves in B represent the elasticity curve (storage modulus, G') over time.

Generally, milk from different breeds showed large differences in coagulation properties, with Danish Jersey having superior coagulation abilities. Distribution of RCT and CFR in the individual milk samples from Danish Holstein and Danish Jersey is shown in Figure 7 and in Table 13. All milk samples having SCC > 500.000 cells/ml or non-coagulating milk properties in duplicate measurements were excluded from these reportings, as outlined in Poulsen et al. (2013).

A



B

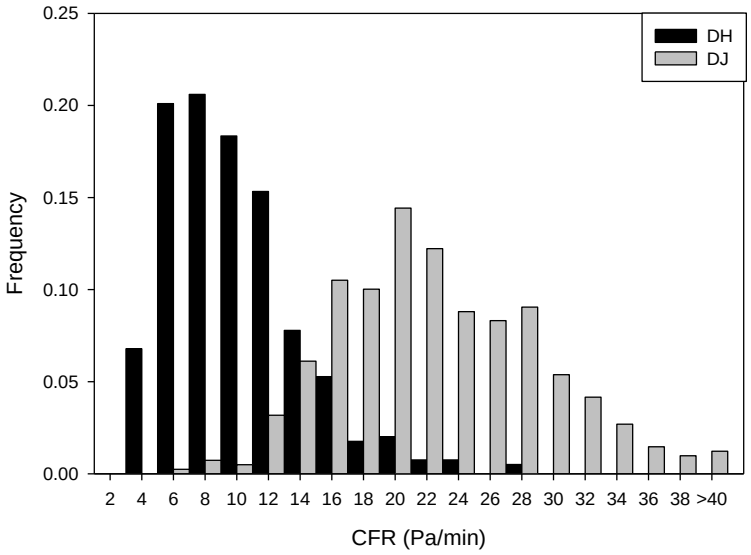


Figure 7. Distribution of (A) Rennet Coagulation Time (RCT) and (B) Curd Firming Rate (CFR) in Danish Holstein (DH) and Danish Jersey (DJ). RCT and CFR are without non-coagulating milk samples in one or both coagulation measurements. Adapted from Poulsen et al. (2013).

For RCT, Danish Holstein and Danish Jersey showed somewhat overlapping distributions, but with Danish Holstein having significantly longer RCT, compared with Danish Jersey (Table 13). For CFR, Danish Jersey had significantly higher CFR compared with Danish Holstein and mean CFR was more than double in milk from Danish Jersey relative to Danish Holstein (Poulsen et al., 2013). Cows in second and third parities had significantly higher CFR, than cows in first and fourth parities; and days in milking (DIM) was furthermore positively associated with CFR. In

contrast, RCT was not significantly influenced by neither parity nor DIM (data not shown, Poulsen et al., 2013).

Table 13. Descriptive statistics for milk composition and coagulation traits in Danish Holstein (DH), and Danish Jersey (DJ). Adapted from Poulsen et al. (2013).

	DH (n=417)				DJ (n=408)			
Trait	Mean	SD	Min	Max	Mean	SD	Min	Max
RCT (sec)[1]	1013[a]	202	427	1972	944[b]	141	682	1805
CFR (Pa/min)[1]	8.97[a]	4.18	2.70	26.55	21.63[b]	6.66	5.8	57.30

Superscripts within a row indicate significant differences among breeds ($P < 0.05$)
[1]Descriptive statistics for rennet coagulation time (RCT) and curd firming rate (CFR) are excluding milk samples with reported non-coagulation in one or both coagulation measurements.

Prevalence of non- and poor coagulation

The number of non-coagulating milk samples varied among breeds, with 8 non-coagulating milk samples observed out of 456 in Danish Holstein, whereas no non-coagulating milk samples were detected in Danish Jersey. Thereby 2% of the Danish Holstein cows were categorised as non-coagulating, and comprised samples that did not coagulate in two replicate analyses within 1h after chymosin addition, and therefore such samples did not have any recordable RCT and CFR. In some breeds, impaired coagulation properties of individual milk samples have high prevalence (Tyrisevä et al., 2004; Wedholm et al., 2006; Hallén et al., 2007; Cassandro et al., 2008), and Ikonen et al. (2004) reported 13% of Finnish Ayrshire cows to be producing NC milk. However, it is important to emphasize that no formal definition of NC milk exists, and different studies have been conducted using different rheological methods with different pre-treatments and rennet concentrations. Using the ReoRox method and identical conditions as described here for Danish Holstein and Danish Jersey, 16 % of the samples from Swedish Red were found to be non-coagulating (Poulsen et al., 2013), compared with 2 % for Danish Holstein and 0 % for Danish Jersey. In Swedish Red, especially low ionic and total calcium were found to be associated with non-coagulating milk (Gustavsson et al., 2014b). Therefore, a substantial proportion of these cows seem to produce raw milk unsuitable for cheese production, and the severe coagulation problems in the Swedish Red breeding population are certainly not desirable for the dairies. To potentially alleviate these negative effects on milk coagulation and subsequent gel-formation, effect of blending good, poor and non-coagulating milk was tested on milk samples from individual Danish Holstein-Friesian cows. The coagulation properties of good coagulating (GC) milk were found to be reduced, when blending NC milk and GC milk, in a fashion imitating the principle of titration (Frederiksen et al., 2011a). In addition, at the ratio of 1:4 of NC milk to GC milk, the quality of the milk was noticeably reduced; the effect of blending increasing amounts of PC milk with GC milk had a similar effect on overall milk quality. Exploration of pooling different ratios of GC milk to PC milk suggested that a minimum of 25% must be constituted by GC milk, to impede the negative effects of NC milk on milk quality

(Frederiksen et al., 2011a). However, the impaired coagulation properties of PC milk were able to be improved by addition of calcium chloride, thus enabling the use of (limited amounts of) PC milk in the production of e.g. cheese (Hallén et al., 2010). In NC milk, the enzymatic coagulation (first) phase does not seem impaired, as NC samples and those with poor and good coagulation properties have been shown to have the same caseino-macropeptide content after rennet-induced coagulation (Frederiksen et al., 2011a).

Influence of genetic variants

It is well-documented, that overall protein composition and milk coagulation are partly influenced by the milk protein genes (Lundén et al., 1997; Ikonen et al., 2004). The influence of additive genetic variation on coagulation properties and resulting cheese-making capacity has been documented, e.g. for β-LG, β-CN, and κ-CN genetic variants (Marziali and Ng-Kwai-Hang, 1986; Ikonen et al., 1999a; Wedholm et al., 2006; Hallén et al., 2007; Meza-Nieto et al., 2007). Especially cows with variant B in the κ-CN gene (*CSN3*) have been consistently associated with milk with desirable coagulation properties by exhibiting increased curd firmness. This results from higher CN and protein contents (Ikonen et al., 1997; Mayer et al., 1997; Buchberger and Dovc, 2000; Hallén et al., 2007; Jõudu et al., 2007). The effect exerted by the genetic variants can be due to both presence of different amino acids in the protein backbone resulting from mutations in the coding regions, and/or be the result of different expression of proteins due to accompanying mutations in the regulatory parts of the genes. In Danish Holstein and in Danish Jersey the association between the genetic variants and milk coagulation properties were confirmed (Table 14). *CSN1S1* (coding for α_{S1}-CN) variant C had significantly shorter RCT and higher CFR compared to *CSN1S1* B in Danish Jersey. Likewise *CSN3* (coding for κ-CN) variant B had significantly shorter RCT and higher CFR, compared to *CSN3* A in both breeds, whereas *CSN3* E, which is only found in Danish Holstein, did not have milk coagulation properties that were different from *CSN3* A. For βCN, *CSN2* A^2 and *CSN2* I variants had significantly longer RCT, and for *CSN2* A^2 also significantly lower CFR compared to *CSN2* A^1, whereas *CSN2* B had significantly shorter RCT and higher CFR than *CSN2* A^1 in Danish Holstein. For Danish Jersey, *CSN2* A^2 and *CSN2* I had significantly lower CFR compared to *CSN2* A^1. In Swedish Red, there was a higher frequency of β-CN A^2 and κ-CN A and E protein variants in cows producing non-coagulating milk, compared to the rest of the sample population (Poulsen et al., 2013).

Table 14. Least squares estimates (± standard error) of the effect of CN variants within Danish Holstein and Danish Jersey on rennet induced coagulation properties. Significance level is shown for the effect of CSN1S1 C compared to CSN1S1 B, CSN3 B and CSN3 E compared to CSN3 A, and CSN2 A^2, CSN2 A^3, CSN2 B, and CSN2 I compared with CSN2 A^1 for rennet coagulation time (RCT) and curd firming rate (CFR). Table adapted from Poulsen et al. (2013).

Trait	Allele	Danish Holstein	Danish Jersey
RCT (s)	*CSN1S1* B	1072.27 ± 112.62	1049.55 ± 84.49
	CSN1S1 C	1028.02 ± 101.49	1024.85 ± 10.84*
	CSN3 A	1095.44 ± 109.45	1163.43 ± 83.86
	CSN3 B	1016.67 ± 16.75***	1089.31 ± 12.56***
	CSN3 E	1059.97 ± 27.69	-
	CSN2 A^1	977.38 ± 111.96	1040.84 ± 89.67
	CSN2 A^2	1040.41 ± 16.28***	1053.84 ± 18.40
	CSN2 A^3	974.14 ± 95.77	-
	CSN2 B	882.67 ± 35.18**	998.95 ± 21.32
	CSN2 I	1036.97 ± 28.89*	1084.18 ± 25.82
CFR (Pa/min)	*CSN1S1* B	2.81 ± 2.36	12.88 ± 3.28
	CSN1S1 C	1.62 ± 2.33	14.39 ± 0.42***
	CSN3 A	1.36 ± 2.13	7.28 ± 3.07
	CSN3 B	4.38 ± 0.33***	11.71 ± 0.47***
	CSN3 E	1.21 ± 0.54	-
	CSN2 A^1	5.12 ± 2.33	18.11 ± 3.56
	CSN2 A^2	3.77 ± 0.34***	15.79 ± 0.77**
	CSN2 A^3	3.14 ± 2.17	-
	CSN2 B	6.92 ± 0.75*	17.22 ± 0.85
	CSN2 I	5.77 ± 0.62	15.43 ± 1.08*

*P < 0.05, **P < 0.01, ***P < 0.001.

Influence of calcium

Jensen et al. (2012b) performed analyses on a subset of milk samples exhibiting extremes in NC, PC and GC properties within breeds as defined by coagulation class: good, poor, non (Jersey samples: good n = 27, poor n = 25; Holstein-Friesian samples: good n = 26, poor n = 18, non n = 6). The samples were selected based exclusively on the rheological analysis using a combination of rennet coagulation time and curd firming rate and selected as extremes separately within each breed Table 15 shows the mineral (Ca, P, Mg) compositions relative to coagulation class and breed. The overall trend was towards lower amount of minerals in milk with impaired coagulation properties for both breeds, compared with well coagulating milk. No significant mineral differences were observed between NC and PC Danish Holstein milk. For Danish Jersey milk, significantly lower amounts of total Ca, micellar Ca, micellar P, and micellar P relative to total P were observed in poorly coagulating milk. For Danish Holstein, significantly

lower amounts of minerals in both poorly coagulating and non-coagulating milk were total Ca, micellar Ca, micellar P, and micellar Mg, compared with good coagulating.

Table 15. Mineral composition (LS-mean ± standard error) as function of milk coagulation classes (good, poor and non) and breed. Table adapted from Jensen et al. (2012b).

	Jersey			Holstein-Friesian					
	Good	Poor	Sign.	Good	Poor	Non	Sign.		
Minerals	N=27	N = 25	Good vs. Poor	N=26	N=18	N=6	Good vs. Poor	Good vs. Non	Poor vs. Non
Total Ca (mg/kg)	1632.0 ± 23.0	1457.9 ± 24.0	***	1271.4 ± 21.8	1120.2 ± 26.2	1062.3. ± 45.4	***	***	N.S.
Soluble Ca (mg/kg)	534.1 ± 25.7	518.0 ± 26.7	N.S.	414.3 ± 12.5	383.4 ± 15.0	358.6 ± 26.0	N.S.	N.S.	N.S.
Micellar bound Ca (mg/kg)	1097.9 ± 24.7	939.9 ± 25.7	***	857.1 ± 23.7	736.7 ± 28.5	703.8 ± 49.3	**	**	N.S.
Micellar Ca relative to total Ca	66.4 ± 1.6	64.4 ± 1.6	N.S.	67.2 ± 0.2	65.7 ± 0.3	65.9 ± 1.6	***	N.S.	N.S.
Fraction of micellar bound Ca normalised to total CN									
Total P (mg/kg)	1189.7 ± 18.0	1196.4 ± 18.7	N.S.	967.6 ± 23.3	892.8 ± 28.0	922.7 ± 48.4	N.S.	*	N.S.
Soluble P (mg/kg)	492.1 ± 12.0	558.3 ± 12.4	***	440.8 ± 12.7	432.6 ± 15.3	483.6 ± 26.5	N.S	N.S.	N.S.
Micellar bound P (mg/kg)	697.6 ± 15.6	638.1 ± 16.2	**	526.8 ± 16.5	460.2 ± 19.8	439.1 ± 34.3	*	*	N.S.
Micellar P relative to total P	58.7 ± 0.9	53.2 ± 0.9	***	54.2 ± 0.2	51.6± 0.2	47.2 ± 1.4	*	N.S.	N.S.
Fraction of micellar bound P normalised to total CN									
Total Mg (mg/kg)	128.8 ± 2.4	128.2 ± 2.6	N.S.	111.1 ± 2.3	105.5 ± 2.8	98.4 ± 4.8	N.S.	*	N.S.
Soluble Mg (mg/kg)	90.2 ± 2.3	91.8 ± 2.4	N.S.	80.3 ± 1.8	79.4 ± 2.2	74.3 ± 3.8	N.S	N.S	N.S.
Micellar bound Mg (mg/kg)	38.6 ± 1.6	36.3 ± 1.6	N.S.	30.8 ± 1.3	26.1 ± 1.6	24.1 ± 2.3	*	*	N.S.
Micellar Mg relative to total Mg	30.2 ± 1.0	28.1 ± 1.0	N.S.	27.62 ± 0.2	24.80 ± 0.3	24.22 ± 1.0	***	N.S.	N.S.

Influence of protein composition

The influence of protein composition on rennet coagulation properties was explored within both breeds based on four classes for curd firming rate; non, poor, average and good (Poulsen et al., 2016a). The results revealed differences between breeds as a higher content of κ-CN relative to total protein and higher content of glycosylated κ-CN to total κ-CN were both associated with improved milk coagulation in Danish Holstein. In contrast, lower content of α-lactalbumin was associated with good milk coagulation properties in Danish Jersey, whereas a higher fraction of α_{S1}-CN 8P relative to total α_{S1}-CN was associated with good coagulation (Poulsen et al., 2016). The study thus confirmed the results by Jensen et al. (2012b) on the coagulation subsets. In both studies no difference in protein composition were observed between non- and poor-coagulating milk. Thus, the studies suggest that the aggregation phase seems to be influenced by the degree of phosphorylation, as the more highly phosphorylated forms of α_{S1}- and α_{S2}-CN

are associated with NC and PC milk. Association between lower κ-CN content and larger CN micelles have been confirmed by others (Hallén et al., 2010; Frederiksen et al., 2011a), and also that smaller micelles aggregate faster and form firmer gels (Glantz et al., 2010).

Effect of overall milk composition

Table16 shows milk composition in relation to coagulation class and breed, as determined using Milkoscan analysis of all individual milk samples in the subset (Jensen et al., 2012b). For both breeds, significantly lower contents of total protein and total CN were identified in both PC and NC milk compared with milk of good coagulation ability. In Danish Jersey milk, a significantly lower pH was identified in PC milk, whereas in PC and NC Danish Holstein milk, a significantly lower fat content was identified, while the CN number was higher in PC Danish Holstein milk. No differences in milk composition between PC and NC Holstein milk were found (Jensen et al 2012b). These results are overall in line with the results in Poulsen et al. (2016) using multiple linear regression on the full data set. Generally, milk compositional traits explained only a low proportion of the variation in CFR (R^2 = 0.30 in DH and 0.34 in DJ) and in RCT (R^2 = 0.07 in DH and 0.18 in DJ. In particular, protein content influenced CFR. Furthermore, lactose, citric acid and pH were positively affecting CFR in DJ, but not significantly in DH. For RCT, an increasing pH of the synthesized milk was related to shorter RCT in both breeds. Further also increasing contents of citric acid and lactose were associated with shorter RCT in DJ cows, and protein content affected RCT positively in DJ. These results contrasted earlier findings by Sundekilde et al. (2011), as observed for a limited number of Holstein and Jersey milk samples, where citrate was found to be associated with impaired MCP. Like citric acid, increasing lactose contents in DJ had a positive effect on CFR and a negative on RCT. The positive association of lactose on MCP has also been reported by others (Glantz et al., 2010b; Bland et al., 2014).

Table 16. Milk composition (least square mean ± standard error) as a function of milk coagulation classes and breed. Table adapted from Jensen et al. (2012).

	Danish Jersey			Holstein-Friesian					
	Good	Poor	Sign.	Good	Poor	Non		Sign.	
pH	N=27	N = 25	Good vs. Poor	N=26	N=18	N=6		Good vs. Poor	Good vs. Non
pH	6.7 ± 0.01	6.6 ± 0.01	**	6.7 ± 0.02	6.7 ± 0.02	6.7 ± 0.04	N.S.	N.S.	N.S.
Conductivity (mS/cm)	5.4 ± 0.1	5.6 ± 0.1	N.S.	5.7 ± 0.1	5.9 ± 0.1	5.8 ± 0.2	N.S.	N.S.	N.S.
Fat (g/100 g)	6.1 ± 0.2	5.9 ± 0.2	N.S.	4.4 ± 0.2	3.7 ± 0.2	3.5 ± 0.3	**	*	N.S.
Protein (g/100 g)	4.5 ± 0.1	4.2 ± 0.1	***	3.7 ± 0.04	3.3 ± 0.05	3.3 ± 0.09	***	***	N.S.
CN (g/100 g)	3.1 ± 0.01	2.9 ± 0.02	***	2.8 ± 0.02)	2.6 ± 0.02	2.5 ± 0.04	***	***	N.S.
CN number	70.4 ± 0.003	69.9 ± 0.003	N.S.	75.6 ± 0.01	77.7 ± 0.01	77.8 ± 0.01	**	N.S.	N.S.
Lactose (g/100 g)	4.6 ± 0.03	4.6 ± 0.04	N.S.	4.8 ± 0.03	4.7 ± 0.04	4.7 ± 0.07	N.S.	N.S.	N.S.
Citrate g/kg)	1.86 ± 2.8	1.79 ± 2.9	N.S.	1.62 ± 0.01	1.75 ± 0.01	1.73 ± 0.01	N.S.	N.S.	N.S.

Risk factors for non- and poor coagulation

Important risk factors for NC and PC milk have emerged from these studies, and the results obtained are summarized in Figure 8 and in Table 17. As mentioned earlier, for the Danish Holstein and Danish Jersey breeds, it is noted that the ranges of RCT and CFR used to define GC and PC milk samples differed between the breeds. The NC milk samples obtained from Danish Holstein were defined by no recordable RCT within 60 min and no CFR.

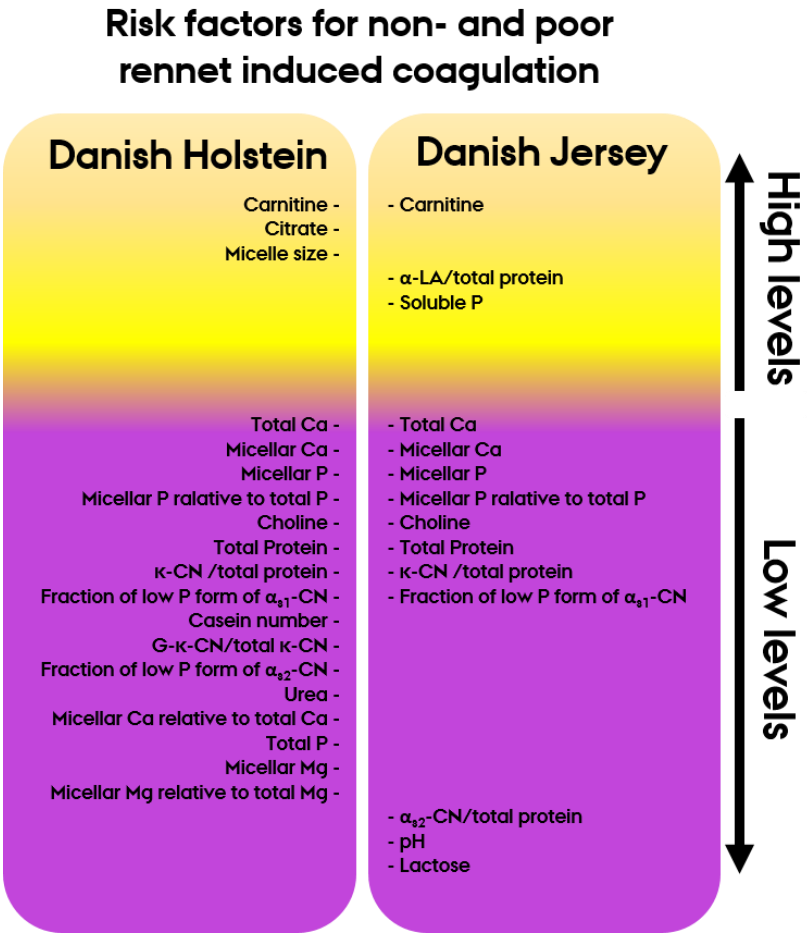


Figure 8. Summary of risk factors for NC and PC milk in Danish Holstein and for PC milk in Danish Jersey determined as associations of individual components with poor rennet induced milk coagulation (based on extreme samples per breed). High (yellow) and low (purple) indicate whether high or low amounts of risk factors are associated with NC/PC milk.

Table 17. Milk compositional risk factors found to be associated with increasing risk of NC and/or PC milk based on published knowledge from extreme samples per breed. Upwards arrows indicate a positive association with NC/PC, while downwards arrows indicate a negative association with NC/PC.

Trait[1]	NC/PC		Reference
	Danish Holstein	Danish Jersey	
Total protein	↓	↓	Frederiksen et al. (2011a) Jensen et al. (2012b)
CN number	↓		Frederiksen et al. (2011a)
Lactose		↓	Sundekilde et al. (2011)
Urea	↓		Frederiksen et al. (2011a)
Citrate	↑		Frederiksen et al. (2011a) Sundekilde et al. (2011)
κ-CN/total protein[3]	↓	↓	Frederiksen et al. (2011a Jensen et al. (2012b) Poulsen et al. (2016)
Glycosylated-κ-CN/total κ-CN	↓		Jensen et al. (2012b) Poulsen et al. (2016)
α-lactalbumin/total protein		↑	Poulsen et al. (2016)
α_{S2}-CN/total protein		↑	Jensen et al. (2012b)
Fraction of low P form of α_{S1}-CN	↓	↓	Frederiksen et al. (2011a) Jensen et al. (2012b) Poulsen et al. (2016)
Fraction of low P form of α_{S2}-CN3	↓		Frederiksen et al. (2011a)
Total Ca	↓	↓	Jensen et al. (2012b)
Micellar Ca	↓	↓	Jensen et al. (2012b)
Micellar Ca relative to total Ca	↓		Jensen et al. (2012b)
Total P	↓		Jensen et al. (2012b)
Soluble P		↑	Jensen et al. (2012b)
Micellar P	↓	↓	Jensen et al. (2012b)
Micellar P relative to total P	↓	↓	Jensen et al. (2012b)
Micellar Mg	↓		Jensen et al. (2012b)
Micellar Mg relative to total Mg	↓		Jensen et al. (2012b)
Choline	↓	↓	Sundekilde et al. (2011)
Carnitine	↑	↑	Sundekilde et al. (2011)
Micelle size	↑		Frederiksen et al. (2011a)
pH		↓	Jensen et al. (2012b)

Factors affecting rennet coagulation time and curd firming rate

In Figure 9 and in Table 18 factors found to be significantly associated RCT and/or CFR within each of the analysed breeds have been summarized.

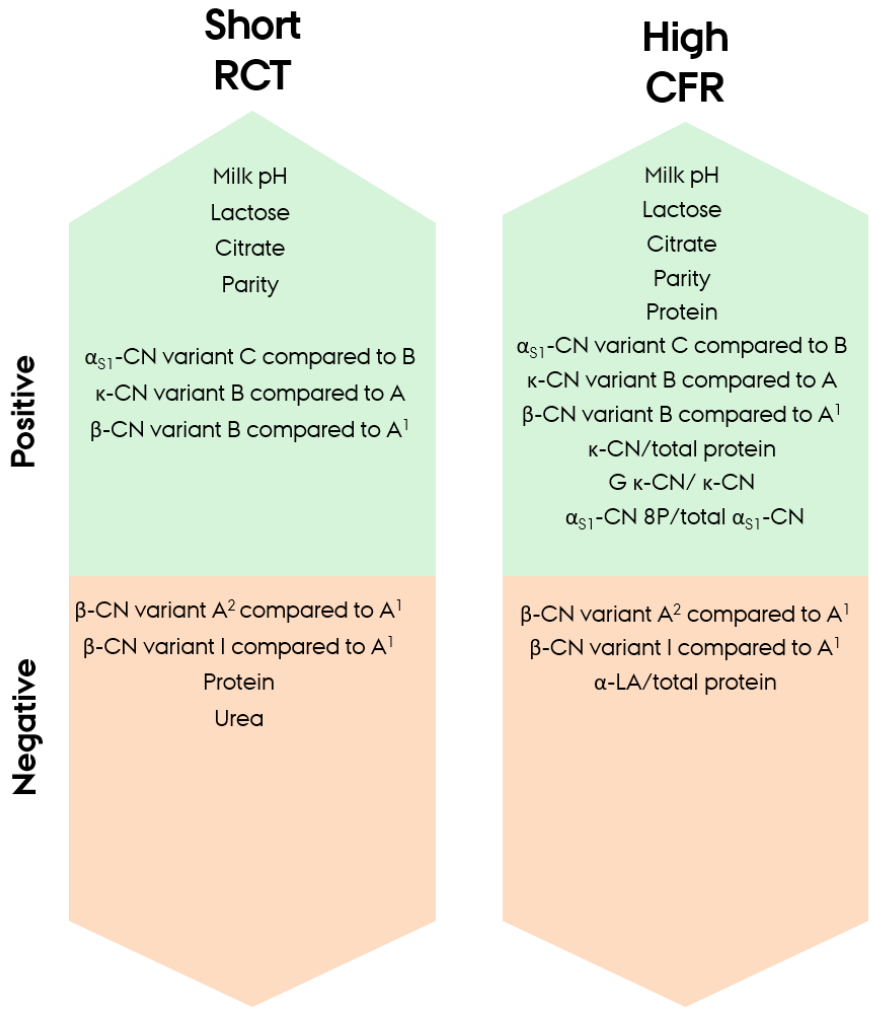


Figure 9. Summary of effects of milk composition on rennet coagulating time (RCT) and curd firming rate (CFR) of cow's milk. Green arrows indicate positive effects and red arrows negative effects of the listed traits with short RCT and high CFR.

Table 18. Summary effects of milk composition and genetic variants on direction of curd firming rate (CFR) and rennet coagulation time (RCT) in Danish Holstein and Danish Jersey milk. Upwards arrows indicate a positive effect on CFR (high CFR) or RCT (low value) of increasing the value of trait, while downwards arrows indicate a negative effect on CFR/RCT.

	Danish Holstein		Danish Jersey		
Trait	CFR	RCT	CFR	RCT	References
Protein	↑		↑	↑	Poulsen et al. (2015c)
Lactose			↑	↓	Poulsen et al. (2015c)
pH		↓	↑	↓	Poulsen et al. (2015c)
Urea				↑	Poulsen et al. (2015c)
Citrate			↑	↓	Poulsen et al. (2015c)
Parity	↑		↑	↓	Poulsen et al. (2015c)
α_{S1}-CN variant C compared to B			↑	↓	Poulsen et al. (2013)
κ-CN variant B compared to A	↑	↓	↑	↓	Poulsen et al. (2013)
β-CN variant A^2 compared to A^1	↓	↑	↓		Poulsen et al. (2013)
β-CN variant B compared to A^1	↑	↓			Poulsen et al. (2013)
β-CN variant I compared to A^1		↑	↓		Poulsen et al. (2013)
Fraction of κ-CN/Total protein	↑				Poulsen et al. (2016)
Fraction of G κ-CN/total κ-CN	↑				Poulsen et al. (2016)
Fraction of α-LA/Total protein			↓		Poulsen et al. (2016)
Fraction of α_{S1}-CN 8P/total α_{S1}-CN			↑		Poulsen et al. (2016)

LA: α-lactalbumin, P: phosphorylated sites.

4. Heritability estimates

The phenotypic variance is the sum of genetic variance and environmental variance, and the heritability (h^2) is thus the proportion of the phenotypic variance, which can explained by the additive genetic variance. Heritability estimates envisage the potential for changing specific trait through selective breeding. Within breed, variance components were estimated following a linear model:

$$Y_{ijk} = \mu + parity_i + DIM + herd_j + animal_k + e_{ijk} \quad [1]$$

Where Y_{ijk} is the phenotype of individual k in parity i and herd j, μ is the fixed mean effect, parity is a fixed effect (i = 1, 2, 3), DIM is covariate of days in milk (d 129 to 229 in Danish Holstein and d 130 to d 252 in Danish Jersey), herd is a random effect of the herd. G is based on the genomic relationship matrix between the animals (VanRaden, 2008).

The proportion of the total variance explained by genetics was calculated as:

$$h^2_g = \sigma^2_a / (\sigma^2_a + \sigma^2_{herd} + \sigma^2_e) \quad [2]$$

The proportion of the total phenotypic variance, which can be explained by herd was calculated as:

$$h_h = \sigma^2_{herd} / (\sigma^2_a + \sigma^2_{herd} + \sigma^2_e) \quad [3]$$

where σ^2_a was the genetic variance, σ^2_{herd} was the herd variance and σ^2_e was the residual variance.

Relative to the publications published from the Milk Genomics Initiative already, all heritabilites have for this report been recalculated based on the above models. In previous studies, herd variance was rarely estimated, which means that the heritability estimates presented in this report are not completely in accordance with those reported earlier in specific papers. However, as the heritability estimates presented here are all based on the same models they are directly comparable. In this report heritability refers to h^2_g and the proportion of the total variance explained by the herd refers to h_h.

Milk composition

Heritability estimates for yield and overall milk composition were low to high, ranging from 0 for somatic cells in Danish Jersey to 0.68 for citric acid in Danish Holstein and Danish Jersey (Table 19). Standard errors ranged from 0.08 to 0.22. This means that genetics explains 68% of the phenotypic variance for citric acid in Danish Holstein in this data set, whereas number of somatic cells in the current data is mainly affected by non-genetic factors. Danish Jersey had

higher heritabilities for eight of the ten traits analysed. Herd heritabilities ranged from 0.04 for SCC in Danish Jersey to 0.50 for urea in Danish Holstein and Danish Jersey. This means that variation between herds explains around 50% of the variation in urea found in the current data set, which most likely relates to differences in feed. Heritabilities for milk compositional traits have also been reported in Poulsen et al. (2015a).

Table 19. Heritability (h^2_g) and the proportion of the total variance explained by the herd (h_h) of milk composition traits in Danish Holstein and Danish Jersey cows. SE denotes standard error.

	Danish Holstein				Danish Jersey			
Trait	h^2_g	h^2_g SE	h_h	h_h SE	h^2_g	h^2_g SE	h_h	h_h SE
Yield (L)*	0.17	0.12	0.34	0.16	0.26	0.13	0.28	0.12
SCC (cells/mL)	0.09	0.15	0.14	0.07	0	0.08	0.04	0.03
Conductivity (mS/cm)	0.20	0.15	0.16	0.08	0.36	0.14	0.28	0.12
pH	0.34	0.16	0.26	0.12	0.39	0.16	0.18	0.09
Fat%**	0.25	0.17	0.17	0.08	0.36	0.16	0.08	0.05
Protein%	0.41	0.19	0.10	0.06	0.55	0.17	0.21	0.09
Casein%	0.36	0.18	0.10	0.06	0.44	0.16	0.15	0.07
Lactose%	0.40	0.19	0.09	0.05	0.51	0.19	0.07	0.04
Urea (mg/L)	0.10	0.09	0.50	0.24	0.41	0.14	0.50	0.23
Citric acid %	0.68	0.22	0.17	0.08	0.68	0.21	0.06	0.04

*Morning milk yield
** g/100 g

Fatty acids

Estimates of heritability for milk fatty acids are presented in Table 20. Most of the heritability estimates for individual fatty acids were low to moderate, ranging from 0 to 0.23 in Danish Holstein and 0.01 to 0.44 in Danish Jersey. Looking at groups, C6:0-C10:0 had higher heritability (0.21) compared to C12:0-C14:0 (0.03). For the four desaturase indices, the highest heritability was estimated for the C14:0 (0.18) and C16:0 (0.11) indices. For the C18 and CLA indices, heritabilities were estimated to be 0.05 and 0.07, respectively. Despite fairly low heritabilities, h_h were also low to moderate for the individual fatty acids ranging from 0 for C16:1 to 0.28 for C16:0. The highest h_h was found for the n-3/n-6 ratio, suggesting that this may be manipulated through feeding. For Danish Jersey especially C14:0 and C14:1 had high heritabilities together with the C14 and C18 desaturase indices.

The heritabilities for specific fatty acids indicate that it may be possible to alter the composition of fatty acids in bovine milk through selective breeding. Due to the negative correlation between groups of saturated fatty acids and unsaturated fatty acids, it may be possible to reduce the concentration of the less healthy saturated fatty acids and increase the concentration of unsaturated fatty acids. For unsaturated fatty acids, our results suggest, that the largest improvements can be achieved through changes in feeding. However, the heritability results also suggest that some achievement can be reached through selective breeding (Krag et al., 2013).

Table 20. Heritability (h^2_g) and the proportion of the total variance explained by the herd (h_h) heritabilities (with standard errors – SE) for individual fatty acids, groups of fatty acids, n-3/n-6 and desaturase indices in Danish Holstein and Danish Jersey.

	Danish Holstein				Danish Jersey			
Trait[1]	h^2_g	h^2_g SE	h_h	h_h SE	h^2_g	h^2_g SE	h_h	h_h SE
c6:0	0.20	0.15	0.11	0.06	0.14	0.13	0.13	0.07
c8:0	0.23	0.16	0.13	0.07	0.14	0.11	0.24	0.11
c10:0	0.17	0.15	0.14	0.07	0.10	0.10	0.29	0.13
c12:0	0.14	0.15	0.14	0.07	0.09	0.09	0.34	0.15
c13:0	0	0.12	0.15	0.08	0.14	0.09	0.25	0.11
c14:0	0.06	0.13	0.24	0.11	0.39	0.16	0.25	0.11
c14:1	0.22	0.16	0.07	0.05	0.44	0.17	0.16	0.08
c15:0	0	0.10	0.25	0.12	0.24	0.11	0.36	0.16
c16:0	0.06	0.12	0.28	0.13	0.15	0.07	0.62	0.29
c16:1	0	0.12	0	0.02	0.26	0.15	0.13	0.07
c17:0	0	0.15	0.07	0.05	0.09	0.11	0.22	0.10
c18:0	0	0.12	0.21	0.10	0.21	0.11	0.40	0.17
c18:1 trans-11[2]	0	0.15	0.14	0.07	0.24	0.12	0.33	0.14
c18:1 n-9	0.01	0.12	0.21	0.10	0.01	0.10	0.27	0.12
c18:2 n-6	0.16	0.15	0.21	0.10	0.12	0.07	0.50	0.23
c18:3 n-3	0.17	0.14	0.21	0.10	0.11	0.07	0.48	0.22
CLA cis-9, trans-11	0.10	0.15	0.12	0.07	0.14	0.10	0.37	0.17
C6:0-C10:0	0.21	0.15	0.14	0.07	0.05	0.10	0.26	0.11
C12:0-C14:0	0.03	0.13	0.21	0.10	0.21	0.12	0.31	0.13
n-3/n-6[3]	0	0.10	0.33	0.15	0.05	0.03	0.82	0.41
C14index[4]	0.18	0.17	0.01	0.02	0.57	0.19	0.04	0.03
C16index[5]	0.11	0.15	0	0.02	0.25	0.16	0	0.02
C18index[6]	0.05	0.13	0.08	0.05	0.54	0.19	0.11	0.06
CLAindex[7]	0.07	0.16	0.04	0.03	0.01	0.11	0.07	0.04

[1]Fatty acids (FA) were determined by gas chromatography and expressed as weight proportion of total identified FA according to Larsen et al., (2011).
[2]C18:1 trans-11: mixture of C18:1 trans-11 and C18:1 trans-10.
[3]Ratio n3/n6 = (C18:3 cis-9, 12, 15/C18:2 cis-9, 12) × 100.
[4]C14 index = C14:1/(C14:1 + C14:0) × 100.
[5]C16 index = C16:1/(C16:1 + C16:0) × 100.
[6]C18 index = C18:1 cis-9/(C18:1 cis-9 + C18:0) × 100.
[7]CLA index = C18:2 cis-9, trans-11/ (CLA cis-9, trans-11 + C18:1 trans-11) × 100.

Protein traits

The heritability estimates for the protein traits for both Danish Holstein and Danish Jersey are presented in Table 21. For Danish Holstein, high heritability estimates were found for casein percentage (0.53), k-CN (0.94), and β-lactoglobulin (0.61). Danish Jersey generally displayed lower genetic heritability estimates compared to Danish Holstein. With regard to PTM isoforms of specific proteins, Danish Holstein showed a much higher heritability for glycosylated k-CN (0.73) and un-glycosylated k-CN (0.80) compared to Danish Jersey, whereas the heritability for 8P α_{s1}-CN is lower in the Danish Holstein (0.29) compared to the Danish Jersey (0.47). This is also reflected in the phosphorylation degree (PD) for α_{s1}-CN (8P α_{s1}-CN/Total α_{s1}-CN), which was higher in Danish Jersey (0.40) compared to Danish Holstein (0.18), whereas the heritability for the α_{s2}-CN phosphorylation degree for was moderate in both breeds (0.36 and 0.38). The heritability for the glycosylation degree (GD) of k-CN (G-κ-CN/total κ-CN) was moderate in Danish Holstein (0.44), but insignificant from zero in Danish Jersey (Table 21). The h_h for protein

traits in Danish Holstein were low to insignificant, whereas moderate h_h were found for α_{s2}-CN (0.52), together with 9P α_{s1}-CN (0.35) and phosphorylation degree for α_{s1}-CN (0.37). The genetic heritabilities were also reported by Buitenhuis et al. (2016).

Table 21. Heritability (h^2_g) and the proportion of the total variance explained by the herd (h_h) heritabilities (with standard errors – SE) for milk protein fractions as well as individual milk proteins and their isoforms in Danish Holstein and Danish Jersey.

	Danish Holstein				Danish Jersey			
Trait[1]	h^2_g	h^2_g SE	h_h	h_h SE	h^2_g	h^2_g SE	h_h	h_h SE
Casein%	0.53	0.21	0.12	0.06	0.22	0.12	0.16	0.08
8P α_{s1}-CN%	0.29	0.17	0.11	0.06	0.47	0.17	0.20	0.10
9P α_{s1}-CN%	0.33	0.21	0.05	0.04	0.04	0.10	0.35	0.16
Total α_{s1}-CN%[2]	0.28	0.17	0.15	0.07	0.09	0.11	0.20	0.09
8P α_{s1}-CN%/ Total α_{s1}-CN%	0.18	0.18	0	0.02	0.40	0.15	0.37	0.17
Total α_{s2}-CN%[2]	0.09	0.14	0.11	0.06	0.11	0.08	0.52	0.24
11P α_{s2}-CN%/ Total α_{s2}-CN%	0.36	0.19	0.08	0.05	0.38	0.19	0.11	0.06
β-CN%	0.30	0.18	0.03	0.03	0.16	0.11	0.21	0.10
G-κ-CN%	0.73	0.21	0.13	0.07	0.05	0.10	0.26	0.12
U-κ-CN%	0.80	0.23	0.06	0.04	0.18	0.16	0.10	0.05
Total κ-CN%[2]	0.94	0.23	0.06	0.04	0.07	0.11	0.16	0.08
G-κ-CN/total κ-CN	0.44	0.18	0.16	0.08	0.04	0.10	0.22	0.10
β-LG%	0.61	0.22	0.10	0.05	0.20	0.11	0.23	0.11
α-LA%	0.21	0.17	0.15	0.08	0.28	0.13	0.14	0.07

[1] Protein and casein (CN) are expressed as percentage traits (g/100 g milk); α_{S1}-CN, 8P-α_{S1}-CN, 9P-α_{S1}-CN, α_{S2}-CN, 11P-α_{S1}-CN, 12P-α_{S2}-CN, β-CN, κ-CN, G-κ-CN, U-κ-CN, α-lactalbumin and β-lactoglobulin are expressed as % of the total protein.
[2] Total α_{S1}-CN comprises 8P-α_{S1}-CN and 9P-α_{S1}-CN; Total α_{S2}-CN comprises 11P-α_{S2}-CN and 12P-α_{S1}-CN; Total κ-CN comprises G-κ-CN 1P and U-κ-CN 1P.

Heritability estimates for absolute amounts of α-lactalbumin, β-CN and osteopontin are presented in Table 22. The variance parameters are estimated on a combined data set using milk and DNA samples from 663 animals as the Milk Genomics samples were boosted by milk samples from the project "Effect of the metagenome on milk quality and composition", The Danish Milk Levy Fund (2013-2015). The heritability estimates were all low and insignificant from zero. Likewise, the h_h for β-CN and osteopontin were low, whereas the h_h for α-lactalbumin was moderate (0.39). These traits were only recorded in milk from Danish Holstein. The heritability for osteopontin is also reported in Poulsen et al. (2018). The potential for increasing these high-value proteins through selective breeding seems to be limited, whereas the results suggest some potential for changing the absolute output of α-lactalbumin through management and feeding.

Table 22. Heritability (h^2_g) and the proportion of the total variance explained by the herd (h_h) heritabilities (with standard errors – SE) for absolute amounts of α-lactalbumin, β-CN and osteopontin in Danish Holstein.

	Danish Holstein			
Trait	h^2_g	h^2_g SE	h_h	h_h SE
Osteopontin (mg/L)	0.11	0.16	0.11	0.07
β-CN (g/L)	0.05	0.15	0.12	0.07
α-lactalbumin (g/L)	0.08	0.11	0.39	0.20

Coagulation traits

Curd firming rate (CFR) had relatively low heritability in Danish Jersey cows (0.20) compared with the high heritability in Danish Holstein cows (0.76). In contrast, the heritability estimates were more similar for rennet coagulation time (RCT), ranging from 0.15 in Danish Holstein to 0.20 in Danish Jersey (Table 23). The h_h were low in both breeds for both traits. The heritability estimates are also reported by Poulsen et al. (2015).

Table 23. Heritability (h^2_g) and the proportion of the total variance explained by the herd (h_h) heritabilities (with standard errors – SE) of milk composition and coagulation traits in Danish Holstein and Danish Jersey cows.

	Danish Holstein				Danish Jersey			
Trait	h^2_g	h^2_g SE	h_h	h_h SE	h^2_g	h^2_g SE	h_h	h_h SE
CFR	0.76	0.22	0.04	0.03	0.33	0.15	0.17	0.08
RCT	0.15	0.14	0.09	0.05	0.20	0.13	0.23	0.1

Vitamins

Poulsen et al. (2015) reported heritabilities for riboflavin (vitamin B_2) in Danish Jersey and Danish Holstein. The low h_h and high heritabilities for both breeds suggest that the riboflavin content in milk is under significant genetic influence and could be changed through selective breeding. Like riboflavin, vitamin B12 is of microbial origin, and in Dutch Holstein Friesian cows vitamin B12 had an estimated heritability of 0.37, which also suggest that vitamin B12 content in milk can be changed through genetic selection (Rutten et al., 2013).

To our knowledge heritability estimates for α–tocopherol (vitamin E) has not been calculated earlier. The heritability was found to be low in both breeds, whereas the h_h was low to moderate. This suggest that α–tocopherol can partly be changed through management and feeding in line with the results found by Poulsen et al. (2012). In particular, a positive association between the level of α–tocopherol and the share of grass in the diet is well-established (Havemose et al., 2004; Slots et al., 2009; Poulsen et al., 2012).

Table 24. Heritability (h^2_g) and the proportion of the total variance explained by the herd (h_h) heritabilities (with standard errors – SE) for alpha-tocopherol and riboflavin in Danish Holstein and Danish Jersey.

	Danish Holstein				Danish Jersey			
Trait	h^2_g	h^2_g SE	h_h	h_h SE	h^2_g	h^2_g SE	h_h	h_h SE
α–tocopherol	0.20	0.13	0.36	0.17	0.09	0.10	0.25	0.11
Riboflavin (B2)	0.58	0.21	0.13	0.07	0.32	0.17	0.11	0.06

Minerals

Heritability estimates for the minerals in milk from Danish Holstein and Danish Jersey are presented in Table 25. Moderate to high heritabilities were found for Ca, Cu, P and Zn in both Danish Holstein and Danish Jersey. For other minerals determined the results were less consistent, i.e. a high heritability was detected in only one of the two breeds analysed e.g. (K (0.45), Mg (0.70), Mn (0.48)) in Danish Jersey and Fe (0.70) in Danish Holstein. For Na and Se, non-existing to low heritabilities were found in both breeds. The h_h were generally low in both breeds except for Se (h^2_h 0.57 and 0.61, respectively). Even though the standard errors are relatively large in our study, the results are generally in agreement with the heritability estimates for Se, Ca, K, Zn, Mg and P presented by van Hulzen et al. (2009). They also found high heritabilities for Ca (0.57), P (0.62) and Zn (0.41) in first parity Dutch Holstein-Friesian cows, but also for Mg (0.60), K (0.46) as also found here in Danish Jersey. In contrast Se had low heritability (0.20) but high h_h (0.65), which fits well with the high h_h found here. This suggests that Se is mainly affected by management and feeding and dietary manipulation is probably possible as also suggested by others, but also soil and thus plant Se may also play a significant role (Muñiz-Naveiro et al., 2005). The heritabilities for milk minerals were also reported in Buitenhuis et al. (2015).

Table 25. Heritability (h^2_g) and the proportion of the total variance explained by the herd (h_h) heritabilities (with standard errors – SE) for Ca, Cu, Fe, K, Mg, Mn, Na, P, Se, and Zn in Danish Holstein and Danish Jersey.

	Danish Holstein				Danish Jersey			
Trait	h^2_g	h^2_g SE	h_h	h_h SE	h^2_g	h^2_g SE	h_h	h_h SE
Ca	0.49	0.23	0.07	0.05	0.61	0.19	0.08	0.05
Cu	0.65	0.25	0.09	0.06	0.39	0.15	0.18	0.09
Fe	0.70	0.29	0.01	0.03	0.03	0.1	0.02	0.03
K	0.09	0.17	0.12	0.06	0.45	0.19	0.07	0.05
Mg	0.13	0.18	0.19	0.10	0.70	0.21	0	0.02
Mn	0.10	0.15	0.10	0.06	0.48	0.17	0.14	0.07
Na	0	0.17	0.05	0.04	0.14	0.12	0.16	0.08
P	0.39	0.23	0.01	0.03	0.50	0.19	0.05	0.04
Se	0	0.08	0.61	0.3	0.06	0.05	0.57	0.26
Zn	0.63	0.25	0.01	0.02	0.49	0.17	0.09	0.05

Oligosaccharides

Heritability estimates for 15 individual oligosaccharides in Danish Holstein and Danish Jersey are presented in Table 26. In Danish Holstein, high heritabilities were found for 2 Hex 1 HexNAc isomer 1 (0.81), 3 Hex 2 HexNAc (0.67), 4 Hex 1 HexNAc (0.53), LNH (0.52), 3 Hex 6 HexNAc 1 Fuc (0.44), and 5 Hex 4 HexNAc 1 Fuc (0.43). For Danish Jersey, high heritabilities were found for LNH (0.93), LNT (0.78), 2 Hex 1 HexNAc isomer 1 (0.63), 4 Hex 1 HexNAc (0.57), 5 Hex 4 HexNAc 1 Fuc (0.54), 3 Hex 6 HexNAc 1 Fuc (0.53). The heritability estimates for 2 Hex 2 NeuAc, 2 Hex 1 HexNAc isomer 2, and 3'-sialyllactose (3'-SL) were not significantly different from zero in both breeds. A similar observation was found for 6'-sialyllactose (6'-SL) and 4 Hex 4 HexNAc 1 Fuc in Danish Holstein. Generally, the acidic OS, which contain NeuAc, had low to insignificant heritabilities in both breeds. In contrast to the moderate to high heritabilites, h_h were low to insignificant in both breed (h^2_h = 0 - 0.12) suggesting that milk oligosaccharides are hard to change through feeding and management. The heritability estimates are also reported by Poulsen et al. (2019).

Table 26. Heritability (h^2_g) and the proportion of the total variance explained by the herd (h_h) heritabilities (with standard errors – SE) for milk oligosaccharides in Danish Holstein and Danish Jersey.

	Danish Holstein				Danish Jersey			
Trait[1]	h^2_g	h^2_g SE	h_h	h_h SE	h^2_g	h^2_g SE	h_h	h_h SE
2_0_0_1_0 (3'-SL)	0.12	0.18	0	0.03	0.11	0.12	0.11	0.06
2_0_0_1_0 (6'-SL)	0.01	0.13	0.11	0.06	0.21	0.15	0.11	0.06
2_0_0_2_0	0	0.17	0.09	0.05	0.03	0.13	0.05	0.04
2_1_0_0_0 isomer 1	0.81	0.28	0	0.02	0.63	0.2	0.06	0.04
2_1_0_0_0 isomer 2	0.06	0.16	0.07	0.05	0.02	0.11	0.07	0.05
3_1_0_0_0 LNT	0.12	0.18	0	0.03	0.78	0.24	0.01	0.02
3_1_0_0_0 isomer 2	0.28	0.19	0	0.02	0.37	0.17	0.12	0.06
3_2_0_0_0	0.67	0.28	0.02	0.03	0.2	0.14	0.03	0.03
4_1_0_0_0	0.53	0.26	0.04	0.04	0.57	0.2	0.05	0.04
4_2_0_0_0 LNH	0.52	0.23	0	0.02	0.93	0.25	0	0.02
5_4_0_0_0	NA	NA	NA	NA	0.29	0.18	0.05	0.04
3_6_1_0_0	0.44	0.23	0.01	0.03	0.53	0.2	0.03	0.03
4_4_1_0_0	0	0.16	0.03	0.03	0.23	0.16	0.01	0.03
4_5_1_0_0	0.22	0.21	0.03	0.03	0.27	0.16	0	0.02
5_4_1_0_0	0.43	0.23	0.01	0.02	0.54	0.21	0	0.02

[1]Oligosaccharides are represented by their monosaccharide compositions, denoted as Hex_HexNAc_Fuc_NeuAc_NeuGc.

Metabolites

Heritabilities for milk metabolites in Danish Holstein and Danish Jersey are presented in Table 27. In Danish Holstein several metabolites had moderate to high heritabilities (16 out of 38 had heritabilities greater than 0.4). In both breeds choline, citrate, glycerophosphocholine and orotate had high heritabilities (> 0.6). In our study, the heritability for urea was 0.16 in Danish Holstein and 0.39 in Danish Jersey. The heritability for Danish Holstein is comparable with the heritability of 0.14 found by Stoop et al. (2007) in Dutch Holstein-Friesian and the study of Mucha

and Strandberg (2011) (0.16 – 0.18), but lower than the heritability found by Miglior et al. (2007) (0.38-0.41), which is, however, comparable with the heritability for in Danish Jersey. For lactose, the heritability was non-existing in both breeds and thus considerably lower compared to the study of Miglior et al. (2007) (0.48-0.51) for Canadian Holstein. The h_h in Danish Holstein were all below 0.3, with creatine having the highest (0.27). In Danish Jersey, the h_h estimates were also generally low with urea having the highest h_h (0.46). The heritability estimates for Danish Holstein are also reported in Buitenhuis et al. (2013), whereas the heritability estimates for Danish Jersey have not been reported before.

Table 27. Heritability (h^2_g) and the proportion of the total variance explained by the herd (h_h) (with standard errors – SE) for milk metabolites in Danish Holstein and Danish Jersey.

	Danish Holstein				Danish Jersey			
Trait	h^2_g	h^2_g SE	h_h	h_h SE	h^2_g	h^2_g SE	h_h	h_h SE
2-oxoglutarate	0.17	0.14	0.15	0.08	0.39	0.19	0.09	0.05
3-hydroxybutyrate (BHBA)	n.e.	0.29	n.e.	0.02	0.34	0.15	0.14	0.07
Acetate	0.19	0.16	0	0.03	0	0.08	0	0.02
Acetone	0.04	0.1	0.21	0.1	0	0.1	0.27	0.12
Alanine	0.44	0.19	0.08	0.05	0.17	0.15	0.07	0.05
Betaine	0.2	0.16	0	0.02	0.05	0.11	0	0.02
Butyrate	0	0.14	0.06	0.04	0	0.1	0.06	0.04
Caprylate	0.26	0.17	0.07	0.05	0.2	0.15	0.04	0.04
Carnitine	0.11	0.13	0.04	0.04	0.08	0.12	0	0.02
Choline	0.62	0.2	0.07	0.05	0.63	0.2	0.09	0.05
cis-Aconitate	0.09	0.13	0	0.02	0	0.1	0.02	0.02
Citrate	0.64	0.21	0.17	0.08	0.65	0.2	0.07	0.04
Creatinine	0.74	0.27	0	0.02	0.11	0.15	0.05	0.05
Fucose	0.66	0.25	0.02	0.02	0.39	0.17	0.03	0.03
Fumarate	0.2	0.14	0.23	0.11	0.47	0.19	0.13	0.06
Galactose	0.53	0.19	0.06	0.04	0.23	0.15	0.03	0.03
Galactose-1-phosphate	0.55	0.21	0.06	0.04	0.35	0.17	0.07	0.04
Glucose	0.58	0.22	0.05	0.04	0.13	0.13	0.05	0.04
Glucose-1-phosphate	0.4	0.2	0.01	0.02	0.11	0.15	0.03	0.03
Glutamate	0.46	0.2	0.05	0.04	0.19	0.17	0.02	0.03
Glycerophosphocholine	0.6	0.21	0.07	0.04	0.71	0.22	0.04	0.03
Hippurate	0.52	0.19	0.19	0.09	0.16	0.1	0.32	0.15
Isobutyrate	0	0.14	0.02	0.03	0.1	0.13	0.01	0.02
Isoleucine	0	0.13	0.02	0.03	0	0.1	0.1	0.06
Lactate	0	0.15	0.12	0.06	0.01	0.09	0.09	0.05
Lactose	0	0.14	0.05	0.04	0	0.12	0.01	0.02
Leucine	0.2	0.15	0	0.02	0	0.12	0.12	0.06
Malonate	0.28	0.19	0.19	0.09	0.02	0.11	0.03	0.03
Methionine	0.34	0.19	0.08	0.05	0	0.12	0	0.04
N-acetyl-carbohydrates	0.79	0.23	0.06	0.04	0.29	0.15	0.06	0.04
O-acetylcholine	0.32	0.15	0.24	0.11	0.31	0.16	0.08	0.05
O-phosphocholine	0.34	0.18	0.12	0.06	0.22	0.15	0.04	0.03
Orotate	0.77	0.21	0.09	0.05	0.64	0.2	0.02	0.03
Pantothenate	0.47	0.2	0.07	0.05	0	0.09	0.18	0.08
Tryptophan	0	0.13	0.02	0.03	0	0.09	0.01	0.03
Urea	0.16	0.12	0.38	0.18	0.39	0.14	0.46	0.2
Uridine	0.08	0.13	0.04	0.03	0.15	0.13	0.04	0.03
Valine	0.03	0.13	0	0.02	0	0.11	0.1	0.07

5. Genome wide association studies

Genome-wide association studies (GWAS) were performed in order to identify genomic regions contributing to the phenotypic trait of interest. The quality parameters used for the selection of single nucleotide polymorphisms (SNPs) and animals in the GWAS were minimum call rates of 80% for individuals and marker loci with minor allele frequencies (MAFs) below 1% were excluded. The SNP positions were based on the *Bos Taurus* genome assembly (Btau_4.0) (Liu et al., 2009). In total 494,984 SNP markers were used in both DH and DJ. Unless stated otherwise, a SNP was considered significant at FDR of $P < 0.1$.

Fatty acids

In total 1,233 significant SNP markers were identified for Danish Holstein, spread over 18 different chromosomes for nine FA traits (C6:0, C8:0, C14:1, C16:1, C18:2 cis-9,trans-11(CLA), C6-C10, C14-index, CLA-index, and fat% (FP)) (Buitenhuis et al., 2014). For C6:0, 32 SNP markers were significant (BTA9, BTA12, and BTA25). For C8:0, one significant SNP was detected on BTA9. For C14:1, 83 significant SNPs were detected (BTA5, BTA7 (GRIA1), BTA8 (SYK), BTA12 and BTA26 (ZFYVE27, CRTAC1, DNMBP, SCD)). SCD explained 12.7% of the total variance. For C16:1, 29 SNPs were distributed over two chromosomes (BTA8, BTA14 (ARHGAP39, CYHR1, CPSF1, DGAT1, SMPD5, GRINA, LOC786966, and FAM83H)). DGAT1 explained 7.8% of the total variance. For CLA, two SNPs were significant on BTA17 (FAT4). For C6-C10, 11 SNPs were detected on BTA9 (SOGA3). For C14-index, 398 significant SNPs were detected, of which 368 on BTA26 (167 SNPs could be assigned to 41 genes). The SNP with the highest –log10 (P-value) was assigned to SCD explaining 20.9% of the total variance. For CLA-index, SNPs were detected on BTA16 (PTPRC). For FP, in total 628 significant SNPs were detected (BTA2 (PARD3B), BTA11, BTA14). The significant SNP markers could be assigned to 30 different genes where DGAT1 was among the significant SNPs. DGAT1 explained 22.8% of the total variance.

In total 1,122 significant SNP markers have been identified for Danish Jersey, spread over 26 different chromosomes for 13 FA traits (C10:0, C12-C14, C13:0, C14:0, C14 index, C15:0, C16:0, C18:0, C18:1n9cis, C18:2n6cis, C18:3n3, CLA and FP). For C10:0, five SNPs were identified (BTA13: GSS, UQCC, and BTA26: SEC31B). For C13:0, seven significant SNPs were identified (BTA5: ACSS3, BTA17: MAPK1). ACSS3 explained 11.8% of the total variance. For C12-C14, three SNPs on BTA15 (CADM1). For C14:0 one significant SNP was detected on BTA15 (CADM1). For C14 index, 308 significant SNPs were identified (BTA14, BTA15, BTA18, BTA23, and BTA26). SNPs on BTA26 could be assigned to 32 genes of which SCD is one of them. SCD explained 66.5% of the total variance. For C15:0, 78 SNPs were significant, spread over nine chromosomes including BTA5 (ACSS3), BTA20, and BTA28. ACSS3 explained 17.7% of the total variance. For C16, 33 SNPs were detected (BTA14, BTA27). DGAT1 was among the significant SNPs on BTA14 explaining 12.5% of the total genetic variance. For C18:0, 80 SNPs were detected (BTA10: TDP, KCNK13, TTC7B, CASC4, and CTTDSPL2, BTA27: SUPT3H, RUNX2). For C18:1n9cis, SNPs were

detected on BTA15 (CADM1). For C18:2n6cis, 191 SNPs were detected spread over 17 different chromosomes including BTA4, BTA9, BTA13, BTA24, BTA4. For C18:3n3, 12 SNPs were detected of which 11 were located on BTA8. For CLA three SNPs were detected (BTA10: KCNH5). For FP, 320 SNPs were found to be significant (BTA11: CAPN14, GALNT14, CLAT1, BTA14). On BTA14 DGAT1 is among the genes assigned to SNPs.

In general, there was little overlap between the QTL found in Dutch Holsteins (Bouwman et al., 2011) and those found in Danish Holstein (Buitenhuis et al., 2014), when comparing QTL for C6:0, C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, C14:1, C16:1, and CLA). For Danish Holstein, significant SNP markers were detected for C14:1, C16:1 and CLA on the same chromosomes as detected by Bouwman et al. (2011). In detail, only markers for C14:1 on BTA7 and BTA26, and markers for C16:1 on BTA14 were overlapping with Bouwman et al. (2011). The SNP markers detected for C10:0, C14:0, C16:0 in Danish Jersey had overlap with the study of Bouwman et al. (2011) on BTA13, and BTA26 for C10:0, BTA15 for C14:0, and on BTA14 and BTA27 for C16:0.

Relative protein composition

GWAS results from major milk proteins and their posttranslational modifications were reported by Buitenhuis et al. (2016). In Danish Holstein, 11,052 SNP markers have been detected for protein percentage (200), CN percentage (193), α_{S2}-CN% (200), β-CN% (2), k-CN% (4,742), β-LG% (166), glycosylated-k-CN% (2,759), un-glycosylated-k-CN% (2,544), 11P-α_{S2}-CN% (244), and 12P-α_{S2}-CN% (2). No significant SNP markers were detected for α_{S1}-CN%, 8P-α_{S1}-CN%, 9P-α_{S1}-CN% and α-lactalbumin%, as well as for PD of α_{S1}-CN% and α_{S2}-CN% and GD for k-CN. In Danish Jersey, 287 significant SNP markers were detected for protein percentage (46), CN percentage (60), α_{S2}-CN% (21), k-CN% (21), and β-lactoglobulin (102). 25 SNP markers were detected for 11P-α_{S2}-CN%. Furthermore, PD for α_{S1}-CN (8P- α_{S1}- CN /total α_{S1}- CN) and GD for k-CN (Glycosylated-k-CN /total k- CN) revealed 11 and 1 SNP markers, respectively. No significant SNP markers were detected for β-CN %, α_{S1}-CN%, un-glycosylated-k-CN, glycosylated-k-CN, 8P- α_{S1}-CN%, 9P-α_{S1}-CN%, 12P- α_{S2}-CN and α-lactalbumin% as well as for PD of α_{S2}-CN%.

In the study by Buitenhuis et al. (2016), there was a profound difference in the heritabilities between Danish Holstein and Danish Jersey (Danish Holstein > Danish Jersey**,** Table 21) for k-CN% related traits . These differences in the heritabilities could potentially explain the difference in the number of QTL detected for glycosylated-k-CN and un-glycosylated-k-CN between Danish Holstein and Danish Jersey. The comparison of the GWAS results for k-CN%, the un-glycosylated-k-CN% and glycosylated-k-CN% revealed a number of glycosylated-k-CN% specific QTL peaks, indicating that there is a potential to genetically differentiate between the glycosylated-k-CN% and un-glycosylated-k-CN% fractions of k-CN in the milk (Figure 10). Among the genes assigned to the significant SNP markers for glycosylated-k-CN, two genes

were related to PTMs of CNs. These genes were: Casein kinase 1, Gamma 3 (*CSNK1G3*) on BTA7 and protein kinase c theta (*PRKCQ*) on BTA13.

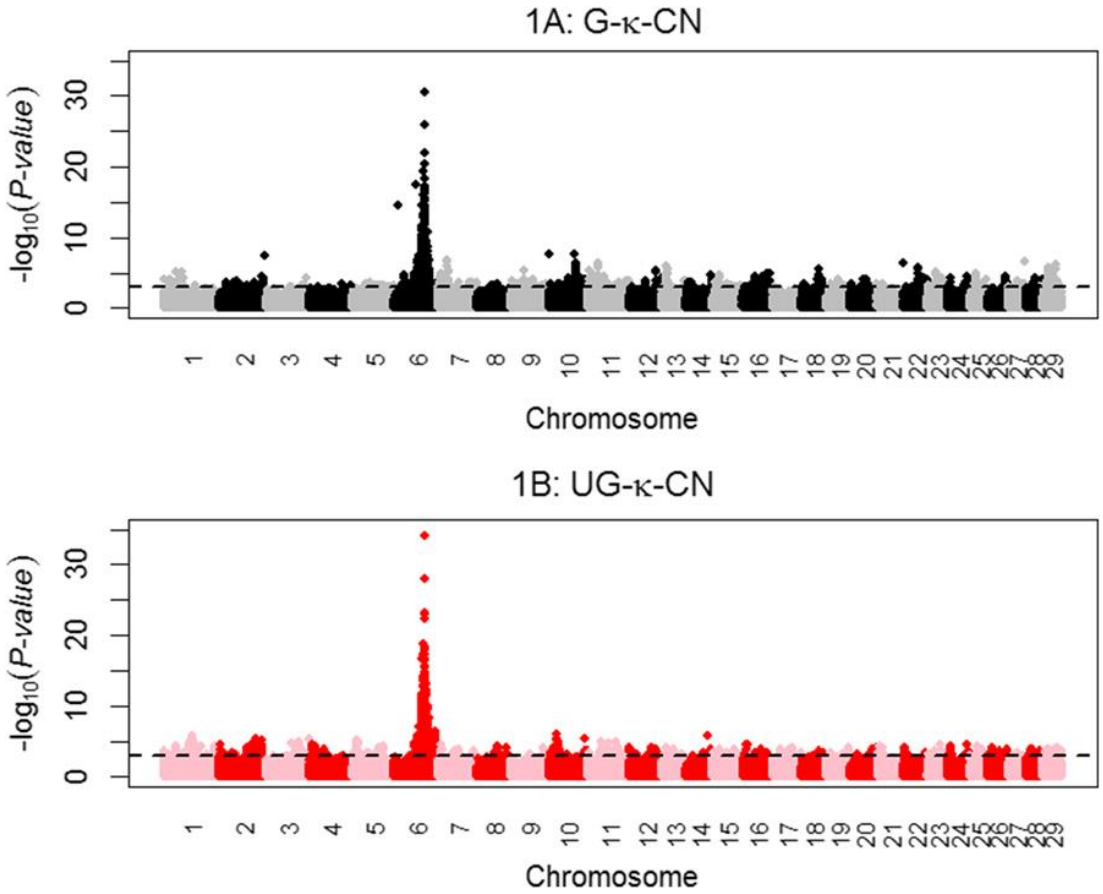


Figure 10. Manhattan plot for G-k-CN (black and grey dots) and U-k-CN (red and pink dots) in Danish Holstein. On the x-axis the chromosomes are represented. On the y-axis the –log10 (P-value) is presented. Adapted from Buitenhuis et al. (2016).

Phosphorylation forms of α_{S1}-CN have earlier been reported to be regulated by different sets of genes (Bijl et al., 2014). In their GWAS study, it was shown that both the 8P and 9P forms of α_{S1}-CN were regulated by a region on BTA6. The 8P form was further affected by a region harboring the *PAEP* gene on BTA11, while the 9P form was additionally regulated by a region harboring *DGAT1* on BTA14 (Bijl et al., 2014). In Buitenhuis et al. (2016), a region on BTA12 was identified for 8P-α_{S1}-CN% in Danish Holstein. Further regions on BTA6 and BTA11 were identified. However, these were not found to be significant.

Coagulation properties

Gregersen et al. (2014) used a GWAS to identify genomic regions affecting RCT and CFR in Danish Holstein. In total, 19 genomic regions on ten different chromosomes affected RCT and CFR. The regions included potential candidate genes of interest e.g. *CSN3* on BTA6, coding for κ-CN, as well as *PAEP* on BTA11, coding for β-lactoglobulin, *CTSD* on BTA29, coding for cathepsin D, as well as potential novel candidate genes such as *CWC15*, *MAP2K5* and *LMAN1*. In Swedish Red, a GWAS for rennet and acid induced coagulation also showed a major QTL

around the casein gene cluster on BTA6 as well as regions encompassing *GALNT1* on BTA24, coding for UDP-N-acetyl-α-D-galactosamine:polypeptide N-acetylgalactosaminyl-transferase 1. These enzymes play a role for O-glycosylation of κ-CN. Also regions containing *CTSZ* on BTA13 and *CTSC* on BTA29 was identified, which support that cathepsins may play a role for milk coagulation, probably due to proteolysis (Gregersen et al., 2015). Poulsen et al. (2017) confirmed the association between haplotypes identified from the GWAS study by Gregersen et al. (2014) and rennet-induced milk coagulation. The identified haplotypes were located within a major QTL on BTA6, where three significant SNPs located in intron regions or downstream *CSN3* were identified, suggesting that the genetic variation is related to the regulation of k-CN.

Vitamins

To our knowledge, Poulsen et al. (2015) was the first to report on GWAS conducted on milk riboflavin content. In Danish Jersey, the GWAS identified 35 significant SNPs associated with riboflavin, whereas in Danish Holstein 511 significant SNPs were identified. In Danish Jersey, significant SNP associations were detected on BTA14, BTA16 and BTA17, and some could be assigned to known genes (*DGAT1*, *HEATR7A*, *PYCRL*, *BAI1*, *TSNARE1*, *FAM135B*, *HMCN1*, and *TMEM132D*). For Danish Holstein, 170 significant SNPs could be assigned to 64 genes, and within these, 55 SNPs were assigned to 19 genes on BTA14. The SNP with the highest -log10(P-value) for Danish Holstein, was located on BTA13 and assigned to *SLC52A3*, which is a riboflavin transporter gene. *SLC52A3* explained 2% of the variation in milk riboflavin content, and could be a good candidate gene responsible for the genetic regulation of riboflavin content in Danish Holstein milk (Poulsen et al., 2015a). In addition, several of the SNPs on BTA14 had very high -log10(P-value) including those assigned to *ARHGAP39*, *CYHR1, CPSF1*, *DGAT1*, *SMPD5*, *GRINA*. Five significant SNPs were overlapping between Danish Holstein and Danish Jersey, and all of these were located on BTA14.

No GWAS has been performed for α-tocopherol in Danish Jersey and Danish Holstein milk.

Minerals

Buitenhuis et al. (2015) reported GWAS results on 10 different minerals in milk from Danish Holstein and Danish Jersey. In milk from Danish Holstein, 649 significant SNP markers were detected for Ca (24), Cu (90), Fe (111), Mn (3), Na (1), P (4), Se (12) and Zn (404). No significant SNP markers were found for K and Mg. In Danish Jersey, 787 significant SNP markers were detected for Ca (44), Fe (43), K (498), Na (4), Mg (1), P (94) and Zn (3). No significant SNP markers were found for Na, Cu, Mn, and Se. One SNP closely linked to the *DGAT1* gene was common between the two breeds for Zn.

Other candidate genes identified in Danish Holstein included *C8H9orf3* for Se and *PDIK1L* for Zn, whereas a linkage disequilibrium (LD) block containing six genes (*TFF1*, *TFF2*, *TMPRSS3*, *UBASH3A*, *RSPH1*, and *SLC37A1*) was associated with P in Danish Jersey. A significant LD block

on BTA 6 for K in Danish Jersey could not be assigned to any genes. *PDIK1L* is involved in biological processes like protein serine/threonine kinase activity, ATP binding and protein phosphorylation. Even though it is not known how this gene plays a role in the regulation of the Zn concentration in the milk at this stage, phosphorylation of the (casein) proteins in milk are important for milk functionalities. In human, *SLC37A1* is involved in the transport of glucose-6-phoshate and plays an important role in the blood glucose homeostasis (Pan et al., 2011). More specifically, *SLC37A1* showed phosphate linked glucose-6-phoshate antiporter activity. *SLC37A1* is part of the SLC37 family. The SLC37 family is a group of genes involved in the translocation of glucose-6-phoshate from the cytoplasm into the lumen of the ER. There, glucose-6-phoshate is hydrolysed into glucose and P (Pan et al., 2011).

Oligosaccharides

A GWAS performed for 15 oligosaccharides identified in total 1770 SNPs for five different OS in Danish Holstein and 6913 SNPs for eleven OS in Danish Jersey (Poulsen et al., 2019). For LNH and LNT in Danish Holstein, a major overlapping QTL on BTA1 explaining 24% of the variation was identified. The two most significant SNPs for LNH had $-\log_{10}$(P-value) of 20.36 (BOVINEHD0100024179) and 20.77 (BOVINEHD0100024184), respectively. For LNT, BOVINEHD0100024179 was also the most significant SNP ($-\log_{10}$(P-value) = 19.77). Interestingly, these SNPs were associated with *B3GNT5*, a gene encoding UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5, a glycosyltransferase involved in glycan synthesis. In Danish Jersey, a highly significant QTL was detected for 2 Hex 1 HexNAc (isomer 1) on BTA11. The most significant SNP had –log10(P-value) of 52.88 (BOVINEHD1100030300) and was assigned to *ABO*, a gene encoding ABO blood group glycosyltransferases. This SNP has been reported to be a missense mutation and explains 56% of the OS variation. Other candidate genes of interest identified for milk OS were *ALG3*, *B3GALNT2*, *LOC520336*, *PIGV*, *MAN1C1*, *ST6GALNAC6*, *GLT6D1*, *GALNT14*, *GALNT17*, *COLGALT2*, *LFNG* and *SIGLEC*. To our knowledge, the study by Poulsen et al. (2019) is the first study to document a solid breeding potential for bovine milk OS and a strong indication of specific candidate genes related to OS synthesis underlying this genetic influence. This new information has the potential to guide breeding strategies to achieve production of milk with higher diversity and concentration of OS and ultimately facilitate large-scale extraction of bovine milk OS.

Metabolites

Buitenhuis et al. (2013) performed a GWAS for 31 individual milk metabolites in Danish Holstein. In total, 29 QTL were detected for 19 different metabolites with SNP associations. Eight of these had genome-wide significance (Bonferroni $P < 0.05$); the other traits had chromosome-wide significance (Bonferroni $P < 0.05$). Three QTL were detected for orotic acid (BTA1, BTA5, BTA19). The most significant SNP for the QTL for orotic acid on BTA1 is located within integrin beta 5 (*ITGB5*), whilst the QTL on BTA5 harboured the phosphodiesterase 3A gene (*PDE3A*) and the QTL at BTA19 harboured the carbonic anhydrase X (*CA10*) gene. The relation of these genes to

orotic acid is unclear. Two QTL were detected for malonate (BTA2 and BTA7). The most significant SNP for the QTL on BTA2 was located between fibronectin 1 (*FN1*) and LOC100848551 (40S ribosomal protein S20-like). The most significant SNP for the QTL for malonate on BTA7 was located close to LOC100296630 (pancreatic progenitor cell differentiation and proliferation factor-like). In addition, one QTL was detected for glucose-1-phosphate (BTA2) and three QTL were detected for galactose-1-phosphate (BTA2, BTA19, BTA20). Galactose-1-phosphate and glucose-1-phosphate were found to have a genetic correlation of -0.36. Galactose-1-phosphate and glucose-1-phosphate exhibited the same SNP peak on BTA2. The most significant SNP for the QTL on BTA2 was located within *RAP1GAP* (RAP1 GTPase activating protein). Furthermore, within the same QTL region, UDP-galactose-4-epimerase (*GALE*) was located. GALE is a UDP-glucose 4-epimerase which catalyses the reaction UDP-glucose into UDP-galactose. Both metabolites, together with glucose and galactose, play an important role in galactose metabolism (KEGG map00052). Two QTL was detected for glucose (BTA6, and BTA11). The most significant SNP for the QTL on BTA11 was located between LOC100848307 (uncharacterised) and *ABO* (ABO blood group). The ABO gene is a glycosyl transferase associated with ABO blood group but its function is relevant to the observed signal.

One QTL was detected for urea (BTA12), where the most significant SNP for the QTL was located between *SERTM1* (serine-rich and transmembrane domain containing 1) and *CCNA1* (cyclin A1). Previously, six QTL have been detected for milk urea nitrogen in Holstein cattle (Bouwman et al., 2010). However, none of these QTL overlapped with the QTL for urea detected in this study on BTA12. Moreover, carnitine and glycerophosphocholine had the same genome-wide SNP peak on BTA25 with a significant SNP located in the *CLN3* gene (ceroid-lipofuscinosis, neural 3), which encodes a protein that is involved in lysosomal function. Both carnitine and glycerophosphocholine play a role in fatty acid metabolism and have a high genetic correlation (-0.59). An additional QTL on BTA29 for was found for glycerophosphocholine. Furthermore, QTLs were detected for N-Acetyl-carbohydrates (BTA1), choline (BTA3, BTA14), glutamate (BTA6), isoleucine (BTA8), an unknown metabolite (unknown 1.6, BTA8, BTA12, BTA29), citric acid (BTA20), fucose (BTA20, BTA23), isobutyrate (BTA24, BTA26) and 2-oxoglutaric acid (BTA26). GWAS for milk metabolites for Danish Jersey has not been conducted.

6. Major QTL regions

A number of major QTL regions associated with specific candidate genes for milk compositional traits have previously been identified. These include the region around *DGAT1* on BTA14, *SCD1* on BTA26, *FASN* on BTA19 as well as the casein gene cluster on BTA6, *PAEP* on BTA 11 and *ALBA* on BTA5. Results related to these genes and regions will be summarised below.

DGAT1

DGAT1 codes for an enzyme that plays an important role in triacylglycerol synthesis by catalysing the esterification of a fatty acyl-CoA to the sn-3 position of a diacylglycerol in the synthesis of triglycerides (Grisart et al., 2002). Effects of the K232A polymorphisms within the gene has been intensively studied (Grisart et al., 2002; Bovenhuis et al 2016), but the gene or the chromosomal region around *DGAT1* on BTA14 has also been shown to affect a range of milk compositional traits, both related to economically important traits, milk metabolites and to milk fatty acid composition (Schennink et al., 2009; Stoop et al., 2009; Buitenhuis et al., 2013; Melzer et al., 2013). Bovenhuis et al. (2016) found that the *DGAT* polymorphism affected the content of several fatty acids (C14:0, C16:0, C15:0, C16:1, C18:1 cis-9, CLA cis-9, trans-11, C18:2 cis-9, cis-12 (n-6) and C18:3 cis-9, cis-12, cis-15 (n-3)) across breeds (Danish Holstein and Danish Jersey). *DGAT1* explains a large part of the variation in the fatty acid composition (Schennink et al., 2007, 2009; Stoop et al., 2009), but also shows a strong effect on milk yield (Schennink et al., 2007). In Buitenhuis et al. (2014), *DGAT1* shows association to C16:1 and fat percentage, explaining 7.8 and 22.8 % of the total variance, respectively, for Danish Holstein. In Danish Jersey, *DGAT1* was associated with C16:0, explaining 12.5% of the total variance. This is in line with the literature, where it has been shown that *DGAT1* is underlying large genetic variation in milk fat composition traits, including milk fat percentage, C14:0, C16:0, and CLA (Schennink et al., 2007). Furthermore, it was shown that *DGAT*1 is associated with desaturation indexes in Dutch Holstein and Italian Brown cattle (Schennink et al., 2008; Conte et al., 2010). In Buitenhuis et al. (2014), we did not find any association with *DGAT1* and desaturation indexes.

Apart from the association to fatty acid traits, *DGAT1* and the chromosomal region on BTA14 was also identified in relation to protein and CN percentage (Buitenhuis et al., 2016). In this study, one of the common, significant SNPs between Danish Holstein and Danish Jersey was also found to be associated with *DGAT1*. If the genome-wide Bonferroni correction for multiple testing was applied, only three major regions for the protein composition would be detected both in Danish Holstein and Danish Jersey milk: BTA6 (k-CN) covering the casein gene complex, BTA14 (protein percentage and CN percentage) covering the *DGAT* gene, and BTA11 (β-lactoglobulin) covering the *PEAP* gene. This is in line with the findings of (Schopen et al., 2011). Interestingly, when analysed as a yield trait, the QTL on BTA14 for protein was not detected

(Schopen et al., 2011). This is in line with the findings of Bovenhuis et al. (2016) who detected significant association with mineral composition in the milk and *DGAT1*, when analysed as a percentage trait, while when analysed as a yield trait, the association with *DGAT1* disappeared. This suggested, that the QTL on BTA14 has an indirect effect on protein and CN percentage (Bovenhuis et al., 2016). BTA14 and *DGAT1* were also positively associated with the genetic regulation of a number of milk traits including riboflavin in Danish Jersey and Danish Holstein.

A SNP marker close to *DGAT* was among the significant markers for Zn in both Danish Holstein and Danish Jersey. How DGAT is involved in the Zn concentration in milk is at this stage unknown; it has, however, been shown that a diet inducing low *de novo* milk fat synthesis can significantly increase plasma Zn and milk Zn content. This indicates, that the transfer of fat from diet to milk might facilitate transfer of Zn from diet to milk (Wiking et al., 2008).

SCD

The enzyme stearoyl-CoA desaturase (SCD1) catalyses the conversion of C10:0 to C18:0 saturated fatty acids into their mono-unsaturated counterparts. The *SCD1* gene is located on BTA26, and is associated with milk fatty acid composition (Mele et al., 2007; Schennink et al., 2008; Conte et al., 2010). In Buitenhuis et al. (2014), associations with *SCD* were detected for C14:1 and C14 index for Danish Holstein and for C14 index for Danish Jersey. Especially, for the C14 index, we found a strong association with *SCD* in both breeds, explaining a large part of the total variance, 20.9 and 66.5% respectively. However, we did not detect any association with *SCD* and the other indices. Mele et al. (2007) and Conte et al. (2010) also detected an association between *SCD* and C14 index, but did not find any association to other desaturase indexes in Italian Holstein and Italian brown cattle, respectively. On the contrary, Schennink et al. (2008) detected an association between *SCD* and six different desaturatase indexes, of which *SCD* explained the largest part of the genetic variation for the C14 index (52%). The size of the study of Schennink et al. (2008) is much larger, compared to our study, as well as to the studies by Mele et al. (2007) and Conte et al. (2010), and therefore has a higher power to detect associations. The association of *SCD* with the C14 index has now been confirmed in three different breeds (Holstein, Italian brown and Danish Jersey), which strengthens the suggestion that the C14 index is the best indicator for *SCD* activity. As C14:0 is almost solely derived from *de novo* synthesis in the mammary gland, it is likely that almost all the C14:1 cis-9 is synthesised by *SCD* (Bernard et al., 2006).

Casein gene cluster

As stated, the BTA6 contains the casein gene cluster, where the genomic organization of the casein loci is spanning 250 kb (Threadgill and Womack, 1990). As 80% of the proteins in bovine milk are caseins, the region is expected to have a large influence on milk protein and related traits. After applying genome-wide Bonferroni correction for multiple testing, a region on BTA6 covering the casein gene complex was still significant for k-CN, both in Danish Holstein and

Danish Jersey milk (Buitenhuis et al. 2016). Likewise, the region has been identified to have strong association to rennet-induced milk coagulation properties in Danish Holstein (Gregersen et al., 2014), as well as to rennet- and acid-induced coagulations in Swedish Red (Gregersen et al., 2015). Poulsen et al. (2017) confirmed the association between haplotypes identified from the GWAS study by Gregersen et al. (2014) and rennet-induced milk coagulation. The identified haplotypes were located within a major QTL on BTA6, where three significant SNPs located in intron regions or downstream *CSN3* were identified suggesting, that the genetic variation is related to the regulation of k-CN.

PAEP

Interestingly the genomic region on BTA11, which harbours the *PAEP* gene, has been associated with milk compositional traits in a number of studies (Gregersen et al., 2014; Poulsen et al., 2018). The gene encodes β-lactoglobulin and thus some relation to this whey proteins would be expected.

Possibilities for genomic improvement

In the Danish Milk Genomics Initiative we studied Danish Jersey and Danish Holstein. They differ phenotypically in their milk compositions, as well as in their genetic make-up, as these breeds have been genetically separated for many generations (Kantanen et al., 2000), and have undergone strong artificial selection. This is also reflected in the difference in the LD structure, where DJ has higher bin-wise LD across the genome (Buitenhuis et al., 2016).

Seven of the 25 most heritable traits were shared between Danish Holstein and Danish Jersey. These included Zn, 2 Hex 1 HexNAc isomer 1, citrate, as well as four metabolites (fucose_overlaps, choline, orotic acid, and N-acetylated-unknown-compound 3). Additionally, six oligosaccharides, five minerals, protein, lactose, four metabolites, 8P- α_{S1}-CN and two desaturase indices were among the top 25 traits in Danish Jersey. In contrast, the most heritable traits in Danish Holstein were ten other metabolites, three protein traits, two minerals, one oligosachharide, and CFR. Zn, citrate and choline are all essential compounds in cellular processes, which may be underlying the strong heritability.

Of the 25 traits having highest herd heritabilities, 15 overlapped between Danish Holstein and Danish Jersey. These included urea, Se, conductivity, and milk yield, together with two metabolites (acetone and hippuric acid), and seven individual fatty acids plus a group of fatty acids (C12-C14) and n-3/n-6 ratio. In addition the results from the GWAS confirmed major QTL regions and identified novel candidate genes for a high number of milk compositional traits.
For implementation of these traits either in the national breeding goal or for milk differentiation at specific farms it is of importance that:

- There is an economic benefit for the farmer and an economic interest for the dairy industry
- There is a method to easily measure these new milk phenotypes at large scale which can be preferable implemented in the current infra structure of the national milk recording system (e.g. FT-IR). This will ensure that breeding values as well as genetic correlations to current breeding goal traits can be established.

7. Technological potential of milk compositional variations

Within Milk Genomics and related projects, the focus has been on healthy cows in mid-lactation within the first three parities, and milk samples have only been collected from conventional farms. Therefore, the variation presented is probably less than the variation in milk, which is weight-in at the Danish dairies, where e.g. seasonal variation may also be pronounced, especially for organic production lines. Likewise, the main technological property explored has been rennet-induced milk coagulation and to some extend acid-induced coagulation, which are the initial steps in cheese and yogurt manufacturing. Nevertheless, this project holds a unique reference for variations in both major and minor milk constituents, which have not been reported to such extent before. This variation will form the backbone for the technological potential presented here, but will also be expanded a bit to summarize more generally on how natural variations in the raw milk may be exploited into future dairy products. The natural variances in milk composition observed and presented in this report, as well as some potentials for different production systems point at possibilities for differentiation and exploitation within the dairy chain, and is presented in Figure 11.

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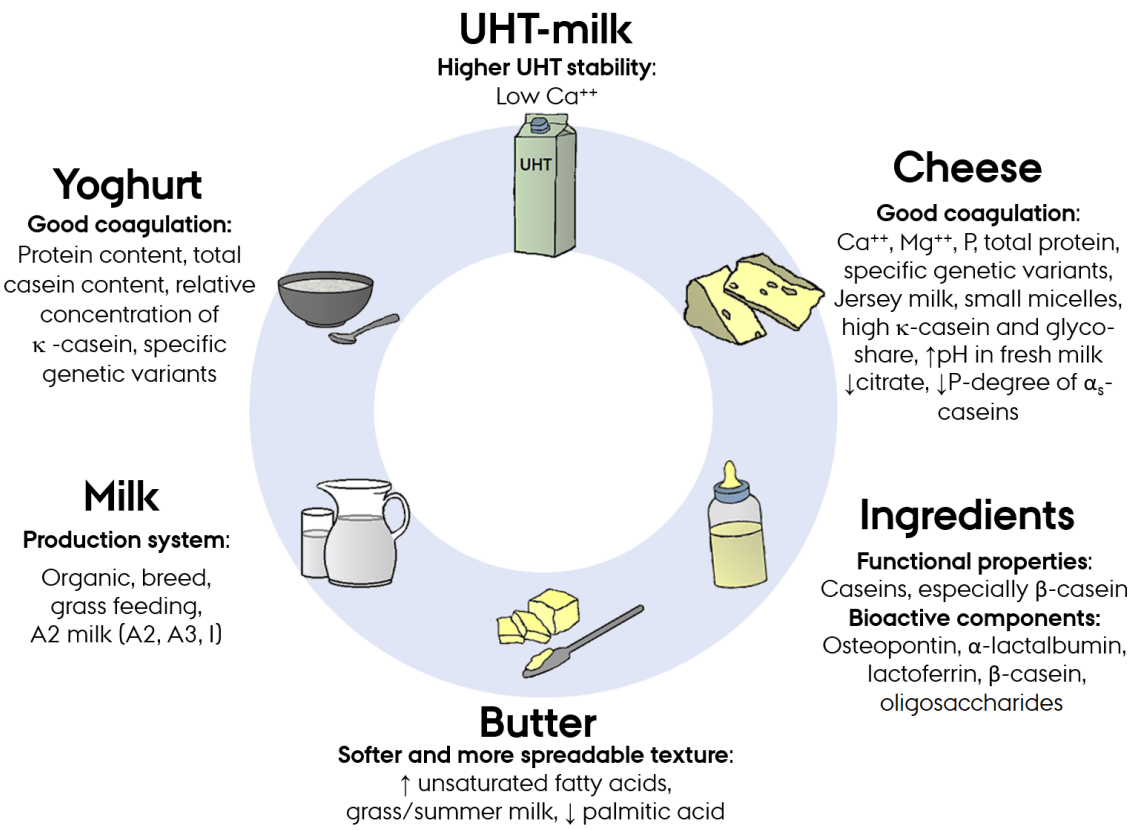

Figure 11. Potentials on utilizing variances in raw milk composition for specific dairy products as based on the traits covered in the present report.

Butter and high fat products

The composition of milk fat from dairy cows is related to both genetic and environmental factors. From a nutritional point of view it is desirable to increase the level of unsaturated fatty acids in milk, which in turn depends on the interplay between the feed composition, rumen metabolism and mammary synthesis. The nutritional value of milk is related to essential fatty acids (linoleic and α-linolenic acid), as well as specific fatty acids showing beneficial health effects, such as conjugated linoleic acid (CLA) and omega-3 fatty acids (German and Dillard, 2006). An increase in healthy fatty acids in human food sources and likewise a decrease in fatty acids with negative impact on human health (in particular palmitic acid) is thus desirable (Mensink et al., 2003; Givens, 2010).

Generally, for butter production and quality, the more unsaturated fat that is present, the softer and more spreadable the butter turns. This is in line with observations by blending in vegetable oils for various spreadable butter blends, and for butter there is a positive correlation between higher levels of unsaturated fatty acids and a softer product, which is desirable among consumers, but may be unwanted from a functionality point of view. This is also in line with a healthier profile linked to unsaturated fatty acids. It is expected though, that a relatively large change in the fatty acid profile is necessary to obtain a significant change in the texture. Apart

from the fatty acid profile and the share of unsaturated to saturated fatty acids, also the fat globule size and the crystallization are important for texture of butter products. It should also be noted, that higher level of unsaturated fatty acids generally also can lead to increased oxidation and potential development of rancid flavour. As vitamin E can have antioxidant activity variances in vitamin E can potentially counteract this effect to some degree, by combining high vitamin E levels with high-unsaturated fatty acid milk for butter production. Elevated vitamin E has been shown to be correlated with feeding of grass products (Poulsen et al., 2012). Furthermore, the n-3:n-6 ratio is higher in milk from grass-fed cows, which is also regarded as **positively associated with health parameters.**

Low pasteurized drinking milk

Consumers milk holds a potential for differentiation, which is also currently exploited with regard to production system (e.g. organic milk, grass milk, unstandardized and/or unpasteurized "farm milk", milk with high n-3:n-6 ratio) or special breeds (Jersey, Holstein, or native breeds) or milk for special segments, like lactose-free milk. Especially, the segment buying lactose-free milk has been growing fast, which has also resulted in the production lactose-free dairy products outside the consumer milk category. Emerging types may include "climate" milk, produced with regard to decreasing the methane emission, through modified feeding regimes, feed additives and/or genetic improvements through breeding towards lower greenhouse gas emission per kg milk produced.

Another area receiving potential increasing interest is the differentiation of drinking milk based on genetic variants of the milk proteins. In the past 20 years there has been a, at times quite intense, debate both in the press and within the scientific community about the potential health related differences between A^1 and A^2 variant of β-CN (see e.g. Truswell, 2005; Larsen, 2014, 2016; He et al., 2017). This has resulted in a range of publications claiming that milk containing the A^2 variant of β-CN gives another physiological response compared to A^1 variant (summarized by Truswell, 2005). β-casein is one of the most prevalent proteins in cow's milk, present in amounts above 9 g/l, corresponding to more than 25% of the total protein content in milk. The difference between the A^1 and A^2 variants of β-CN is an amino acid exchange at position 67, where there in the A^2 variant holds a Proline (Pro), while there in the A^1 variant is a Histidine (His) in this position in the protein sequence. Based on this variation at position 67, the β-CN variants can be grouped into "families", with A^1, B and F variants belonging to the A^1 family and the A^2, A^3 and I belonging to the A^2 family (Table 28), a fact that is often overlooked in the debate and interpretation of frequencies and impacts. It has been reported that the A^2 form is the variant present in human milk and also the oldest, both in humans and cattle (Pal et al., 2015).

Table 28. Amino acid substitutions and organisation into "families" of β-CN variants in DH and DJ. Determined by LC-ESI/MS at protein level. Adapted from Poulsen et al. (2017).

Variant	Amino acid change	A^1/A^2 "family"	DH	DJ
A^1	Pro^{67} His	A1	0.254	0.081
A^2	*reference*	A2	0.621	0.629
A^3	His^{106} Gln	A2	0.004	-
B	Pro^{67} His,	A1	0.044	0.220
F	Ser^{122} Arg	A1	0.008	-
I	Pro^{67} His; Pro^{152} Leu Met^{93} Leu	A2	0.069	0.070

A^1 family: A^1, B and F variants; A^2 family: A^2, A^3 variants

The hypothesis around the A^1/A^2 milk case is that the amino acid exchange from Pro to His at position 67 enables enzymatic release of the peptide called BCM-7 upon digestion. This peptide is released from β-CN variant A^1 (and B and F), but in theory not from A^2 (or A^3 or I), having Pro in the scissile bond. In both Danish Holstein and Danish Jersey, the A^2 variant is the most frequent, but not found exclusively in either breed (Table 28). The BCM-7 peptide is claimed to be linked with stomach discomfort in some persons (Jianqin et al., 2016), but it is still very much debated. The stomach discomfort discussed is resembling the discomfort experienced by lactose intolerants, but recent studies may indicate that some lactose intolerants may have benefits from drinking "A^2" milk instead of milk containing A^1, even when the lactose has been hydrolized (He et al., 2017). The release of BCM-7 peptide by digestion of either purified β-CN or of β-CN in milk from homozygous cows has been studied in two recent studies (Asledottir et al., 2017, 2018). Using a specific method for detection of the peptide in question. It was found that, actually, the BCM-7 peptide was released from not only A^1 family milk, but, surprisingly, also from A^2 family milk, though in lower levels (around 4-5 times lower). It is therefore still a matter of consideration, whether the release of BCM-7 has an impact on gastro-intestinal discomfort in some persons, and whether there is a difference in the significance from A^1 to A^2 milks. Future, controlled studies are needed to settle this.

UHT milk

The composition of milk can have a substantial effect on UHT (ultra high temperature processing) processing and heat stability, as well as affecting storage stability of UHT treated milk. Apart from nutritional effects, milk calcium also has a significant impact on technological properties. Milk calcium, and especially the free ionic form influences stability of long-shelf life UHT milk negatively (Lewis, 2011; Lewis et al., 2011). Currently, the impact of raw milk composition is being investigated on stability of UHT milk in a current PhD project at Department of Food Science, from which more results are expected. Currently, the links between e.g. grass feeding, organic acids and calcium distribution is not well understood. It is suggested that the ionic Ca contributes by neutralizing negative charges on the CN micelles (a.o. influenced by the PTM level), and thereby influence sedimentation processes, both wanted (in cheese making)

and unwanted (in UHT milk). Milk contains in the range of 1.2 g/L (30 mM) total calcium, of which approximately 2/3 is bound to the casein micelles (0.8 g/L, 20 mM), while the rest is found in the whey or serum phase (0.4 g/L, 10 mM). Overall, the total calcium level closely follows the total protein (Bijl et al, 2013), as a tight linkage between total protein to casein and further on to micellar calcium exists. However, the mechanisms governing the calcium distribution in raw milk is less understood. In the literature, there are several interesting reports on additions studies, but it seems that the results and interpretations of those are not directly translatable to understanding why and what governs effects of natural variations in raw milk. For targeted use and understanding of the calcium balances and thereby its impact on UHT product stability,variations In citrate may potentially influence the calcium balance, where especially the level of ionic calcium have been shown to play a potential role for UHT milk stability (Lewis et al., 2011).

Cheese (rennet-induced coagulation)

The PTMs of the CNs affect CN micelle stability, and especially the highly glycosylated, hydrophilic part of κ-CN, the CMP part, ensures electrostatic and steric repulsion between micelles in bovine milk (Dziuba and Minkiewicz, 1996). Several studies have documented that variation in κ-CN content affects casein micelle size (Frederiksen et al., 2011a; Day et al., 2015), but especially the glycosylated part seem to play a major role (Bijl et al., 2014). Thus, casein micelle size was strongly negatively correlated with the content of glycosylated κ-CN, but not with the content of un-glycosylated κ-CN, in milk from Montbéliarde cows (Bijl et al., 2014). Furthermore, CSN3 BB genotypes exhibit higher relative contents of κ-CN (Heck et al., 2009; Jensen et al., 2015a), which seems to be related to higher levels of both un-glycosylated κ-CN and glycosylated κ-CN BB milk relative to κ-CN AA milk (Bonfatti et al., 2014). Apart from the glycosylation of κ-CN, the relative distribution of the caseins, the associated calcium phosphate nanoclusters and phosphorylations of phosphoserine sites in the caseins also affect the technological properties of milk. Thus, a higher fraction of the least phosphorylated forms of α_{S1}-CN and α_{S2}- CN and a higher fraction of glycosylated κ- CN were found to be positively associated with good rennet coagulation properties in milk from Danish Holstein cows (Frederiksen et al., 2011a; Jensen et al., 2012b), suggesting that variation related to PTMs play a significant role determining the technological properties of milk.

In addition, the role of calcium in the casein micelle structure and in the milk coagulation process is fundamental (Holt, 1992; Fox, 2009), and impaired clotting properties have, among others, been linked to a lower content of the main cations in milk, calcium, and magnesium, along with the main anion, inorganic phosphate (Tervala and Antila, 1985; van Hooydonk et al., 1986; Tsioulpas et al., 2007; Frederiksen et al., 2011a), and a higher citrate content (Sundekilde et al., 2011). The minerals in milk exist in dynamic equilibrium between a soluble serum phase and the colloidal micellar phase, and this mineral distribution is influenced by both cooling,

lower milk pH and organic acids like citrate, which all lead to dissolution of calcium phosphate from the micellar phase, which may influence the integrity of the casein micelles and potentially thereby negatively influence the coagulation properties (Gaucheron, 2005). Overall, it seems, that a certain level of calcium is needed to saturate and stabilize the casein micelles, and make them optimal for the cheese making process, while on the other hand, a certain level of ionic calcium is needed in order to diminish the repulsion between the casein micelles in the 2. phase of the rennet induced milk coagulation. Legally, it is allowed to add 20 g $CaCl_2$/100 l milk, corresponding to 0.02 w/w %. Often less is added, e.g. 5 g /100 kg milk corresponding to 0.005 w/w %, or approximately 0.5 mM.

The implications of these well-documented variations in coagulation properties of the raw milk points towards possibilities for improvement of raw milk composition for cheese production. It remains, however, to be established, to which extend these variances in the coagulation properties of the raw milk is manifested in the properties of cheese milk after heat-treatment and addition of starter cultures. This will have to be focus of future studies.

Yoghurt and fermented products (acid induced coagulation)

The addition of acid (lowering the pH towards pH 4.6) eliminates the negative charge of the casein micelle surface, enabling protein gel formation (Hallén et al., 2009; Gustavsson et al., 2014c). Following heat-treatment of milk, denatured whey proteins (predominantly β-lactoglobulin) will associate with κ- and α_{s2}-CNs of the CN micelle.

As with rennet-induced coagulation, several traits related to milk composition (e.g. major milk proteins, fat and lactose content) have been linked to the properties of gels formed by acid-induced coagulation (Lucey and Singh, 1998; Hallén et al., 2009). Genetic parameters for traits related to both acid- and rennet-induced coagulation were investigated in Swedish Red cows as part of the Danish-Swedish Milk Genomics initiative (Gustavsson et al., 2014c). Furthermore, a positive phenotypic correlation between acid-induced coagulation and protein content (including both total CN as well as relative κ-CN concentration) was found. In addition, for acid-induced coagulation, negative correlations were found for gel strength and yield stress with milk yield. However, a general pattern was observed where the observed genetic correlations were stronger than the phenotypic correlations and findings have suggested a weak genetic association between rennet- and acid-induced coagulation properties (Gustavsson et al., 2014c). Using this knowledge on the genetic parameters associated with poor acid-induced coagulation might ultimately benefit the production of yoghurt and fermented dairy products.

Ingredients

The rapidly growing milk ingredient industry exploits the vast nutritional and biological potential of milk. The main drivers are the globalization, demand for high quality nutrients and the stagnation at the European market with simultaneous decline in the demand for traditional yellow cheese. Thus, it is mandatory to explore the possibility of tailoring the content of high value proteins in bovine milk to ensure the most optimal value proposition of milk at the global ingredient market. High value proteins, phospholipids, and oligosaccharides exert nutritional and health promoting effects for consumers. Such value has traditionally been through more traditional dairy products, but a growing market for specialized milk additives and ingredients based on bioactive proteins or proteins with special functionalities (physiologically, technologically) adds great value to the raw milk. An example of this is α-lactalbumin, one of the main proteins in human milk, which is added to infant formula in order to balance the composition towards that of human milk. Other functionally important proteins include one of the major milk proteins, β-CN, as well as the less abundant, but bioactive proteins, like osteopontin and lactoferrin. The reported investigations showed that for osteopontin, β-CN and α-lactalbumin as ingredients in especially infant formulae, large biological variations were revealed, showing potentials for exploiting this to elevate the levels of these valuable components through natural means.

Recently, the production of infant formulae labelled "A2", i.e. containing only the A2 variant of β-casein have gained interest in relation to export markets, where this produce type experiences some demand. The effect of this still remains to be proven. It will, however, be possible to select farms producing this protein type based on typing of the animals. The frequency of A2A2 milk among Jersey cows is slightly higher than among Danish Holstein, but it will not be sufficient to select cows for this based solely on breed, as both breeds represents mixtures and different variants of the β-casein protein, as shown in details above.

OS in milk from Danish Jersey cows contained higher relative amounts of both sialylated and the more complex neutral fucosylated OS, whereas milk from Danish Holstein had a higher abundance of smaller and simpler neutral OS (Sundekilde et al., 2012, Robinson et al., 2018a; Robinson et al., 2018b). The results points at the possibility of exploiting Jersey milk for the enrichment of products with fucosylated oligosaccharides, which are more resembling those in human milk.

8. Summary and perspectives

The phenotypic profiling of the breeds involved (Danish Holstein and Danish Jersey) revealed distinct differences in milk composition in relation to fatty acid composition, coagulation properties metabolites, oligosaccharides, protein composition and the protein modifications (PTMs). The screening of coagulation properties showed that there is large individual differences in these traits between individual cows. The curd firming rate (CFR) was found to be significantly higher for Danish Jersey compared with Danish Holstein. Approximately 2 % of the Danish Holstein samples could not coagulate (were non-coagulating), while up to 17 % were poorly coagulating. In Sweden, 16 % of the Swedish Red milk samples were found to be non-coagulating, which is a surprisingly high proportion, and calls for improvement and elimination. Currently, a project dedicated to that is running in Sweden and led by Lund University.

The distinct milk coagulation properties among breeds were shown to be related to casein gene polymorphisms, and therefore partly a result of the large variation in the number of variants present and their frequencies among breeds. The studies have revealed new genetic variants present in the Danish population of dairy cows, including the C variant of α_{S1}-CN, the I and F variant of β-CN and the E variant of β-CN. These different variants have different impacts on the technological properties.

Apart from the contribution of protein variants and isoforms, eight milk metabolites were found to be in significant different levels according to the milk coagulation status.

For fatty acids pronounced differences were observed, and both feed and herd effects on individual fatty acids were determined profound. Furthermore, we found significant differences between cows in relation to n3:n6 index. The highest h_h was found for the n-3/n-6 ratio, suggesting that this may be manipulated through feeding.

NMR-based metabolomics was able to reflect coagulation properties of milk. Furthermore, several metabolites were found to correlate with somatic cell count in milk, including lactate, acetate and β-hydroxybutyrate.

The great variation in coagulation properties among individual cows and breeds were examined more closely using proteomic techniques to identify genetic variants and isoforms in milk proteins. Briefly, the results showed that the specific genetic polymorphisms and PTMs in major milk proteins were associated with milk with extreme coagulation properties. It was shown that the more phosphorylated forms were more prevalent in the poorly coagulating samples. In addition, PTMs of κ-CN influence cleavage of κ-CN, and thus the release of caseinomacropeptide.

The GWAS and the heritabilities confirmed that milk coagulation is under genetic influence and new genomic regions have been identified both in relation to milk coagulation as well as non-coagulation. Besides a major QTL around the casein gene cluster identified in both the Swedish Red and Danish Holstein, additional regions affecting milk coagulation was identified in close proximity to genes influencing glycosylation and phosphorylation, as well as proteolysis. Therefore, it seems likely that milk coagulation is influenced by these processes as well.

Additional studies have comprised biological effects of different genetic variants of β- and κ-CN, where the anti-oxidative and ACE-inhibitory effects of both intact and digested variants of β-CN were tested, as well as the digestibility of the different genetic variants. The digestion and release of BCM-7 from β-CN A^1 and A^2 milk was also studied, and it was found that the BCM-7 was released from all genetic variants by in vitro digestion, though in much higher levels from β-casein variants representing A^1 family compared with A^2 family.

The project has revealed a large variability in composition of Danish dairy milk, that could potentially be exploited in differentiated products, with specific profile of either oligosaccharides (infant formula, nutraceuticals), protein profile (cheese milk or yoghurt milk), dairy products from specific breeds, as a unique data material has been obtained from this project, where biological variation has been documented and linked to genetic information, and which could potentially be exploited.

From a dairy point of view, the results can be used in the area of milk differentiation, as well as a table material for detailed milk composition. For exploitation of natural variation and its use for production of milk, e.g. at specific farms, it is needed that milk with special properties or components are valued in the payment scheme. In order to enable that it is needed to have fast methods for documentation, like FT-IR or other methods, and there is still a need here for developments. Fast methods have potentials both in relation to test and implementation of new genetic markers for milk quality, as well as being very suitable for the dairies for prediction of milk quality and milk sorting for different purposes, as well as process optimization.

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Appendix 1. Alphabetical list of publications related to the Danish part of the Milk Genomics initiative primarily covered in this report and the parameters they address.

Parameter	Breed and sample size	Reference
Digestion of genetic variants of caseins isolated from homozygotes milk, quantification of BCM-7	Danish Holstein-Friesian (N=3, A^1A^1, A^2A^2, II) β-CN homozygotes	Asledottir et al. (2017)
Digestion of milk from homozygotes, quantification of BCM-7	Danish Holstein-Friesian (N=3, A^1A^1, A^2A^2, II) and native Danish breed (N=1, FF) β-CN homozygotes	Asledottir et al. (2018)
Use of pool-seq analyses for detection of genetic variation affecting milk coagulation properties in Danish Holstein	Danish Holstein (n=456)	Bertelsen et al. (2016)
Effect of DGAT1 K232A polymorphism on fatty acid, protein and mineral composition.	Danish Holstein Friesian (n=456) Danish Jersey (n=436) Dutch Holstein-Friesian (n=2,001)	Bovenhuis et al. (2016)
Genetic parameters (heritability and correlation) and quantitative trait loci for metabolites.	Danish Holstein (n = 371)	Buitenhuis et al. (2013)
Genome wide association study (GWAS) and biological pathway analysis for milk fatty acid composition.	Danish Holstein (n= 456) Danish Jersey (n=436)	Buitenhuis et al. (2014)
Genetic parameters (heritability and correlation)) for minerals (Ca, K, Na, P, Mg, Zn, Fe, Mn, Cu, Se).	Danish Holstein (n = 371) Danish Jersey (n= 321)	Buitenhuis et al. (2015)
Genetic parameters (heritability) for the four caseins, α-lactalbumin and β-lactoglobulin and their PTMs.	Danish Holstein (n=371) Danish Jersey (n=321)	Buitenhuis et al. (2016)
Determination of absolute level of osteopontin in skimmed milk by new ELISA method	Danish Holstein Friesian (N=663)	Christensen et al. (2020)
Use of infrared spectroscopy for quantification of individual fatty acids and exploration of highly collinear reference variables	Danish Holstein (n= 455) Danish Jersey (n=435)	Eskildsen et al. (2014)
Use of infrared spectroscopy for quantification of milk protein composition and coagulation properties	Danish Holstein (n= 426) Danish Jersey (n=406)	Eskildsen et al. (2016)
Proteomic profiling and genetic variants of caseins related to poorly coagulating or noncoagulating milk.	Danish Holstein Friesian (n=20) *NB: 53 at initial screening.	Frederiksen et al. (2011a)
Coagulation properties (rennet coagulation time, coagulum firmness, curd firming rate).	Danish Holstein-Friesian (n=58) Danish Jersey (n=40) Danish Red (n=53)	Frederiksen et al. (2011b)
Estimation of genetic parameter for milk protein composition using multi-trait analysis	Danish Holstein (n=650)	Gebreyesus et al. (2016)
Improved genomic prediction for milk protein composition traits by modeling heterogeneous (co)variances from adjacent-SNP groups	Danish Holstein (n=650)	Gebreyesus et al. (2017)
Use of multi-population GWAS to reveal novel genomic regions and candidate genes for milk fatty acid composition	Danish Holstein (n=675) Dutch Holstein (n=1566) Chinese Holstein (n=784)	Gebreyesus et al. (2019c)
Use multi-population reference and GWAS results for improving reliability of genomic prediction for milk fatty acid composition	Danish Holstein (n=675) Dutch Holstein (n=1566) Chinese Holstein (n=784)	Gebreyesus et al. (2019a)
Use of multi-population datasets for joint genome-wide association and meta-analyses of milk fat acid composition	Danish Holstein (n=614) Dutch Holstein (n=1566) Chinese Holstein (n=700)	Gebreyesus et al. (2019b)
Effect of breed and casein genetic variants on protein profile (measured by capillary zone electrophoresis). Effect of composite genotypes.	Danish Holstein (n=415) Danish Jersey (n=406) Swedish Red (n=392)	Gustavsson et al. (2014a)
2-DGE coupled with MALDI-ToF MS for identification of genetic variants of β-casein, κ-casein and β-lactoglobulin. 2-DGE κ-casein isoform quantification in milk representing extremes in coagulation properties	Danish Holstein-Friesians (n=456) Danish Jerseys (n=436)	Jensen et al. (2012a)
The association of overall milk composition, minerals, protein composition and genetic variants with non-, poor and good milk coagulation. Effect of κ-casein glycosylation degree and α_{S1}-CN and α_{S2}-CN phosphorylation degrees.	Danish Holstein (=50) Danish Jersey (n=52)	Jensen et al. (2012b)
Subset based on κ-casein genotypes (variant A, B and E). Effect of κ-casein post-translational modifications on caseino-macropeptide release rate.	Danish Holstein (=17) Danish Jersey (n=12)	Jensen et al. (2015a)
Heritability and SNP markers for individual fatty acids.	Danish Holstein (n=371)	Krag et al. (2013)
Natural and process-induced PTMs in the major bovine	Review	Le et al. (2017)

milk proteins.		
Joint genome-wide association study for milk fatty acid in Chinese and Danish Holstein populations	Danish Holstein (n=371) Chinese Holstein (n=784)	Li et al. (2015)
Determination of absolute levels of α-lactalbumin and β-casein be multiple reaction monitoring	Danish Holstein Friesian (N=663)	Le et al. (2020)
Digestion of genetic variants of caseins isolated from homozygotes milk, bioactivities of peptides from different genotypes	Danish Holstein-Friesian (N=3, A^1A^1, A^2A^2, II) and native Danish breed (N=1, FF) β-CN homozygotes	Petrat-Melin et al. (2015)
Digestion of genetic variants of caseins isolated from homozygotes milk, bioactivities of peptides from different genotypes	Danish Holstein-Friesians and Danish Jersey (N=3, AA, BB and EE) κ-CN homozygotes	Petrat-Melin et al, (2016)
Digestion of genetic variants of caseins isolated from homozygotes milk, bioactivities of peptides from different genotypes	Danish Holstein-Friesian (N=4, A^1A^1, A^2A^2, BB, II) β-CN homozygotes	Petrat-Melin et al. (2017)
The effect of feed and herd on milk fatty acid composition and α-tocopherol content.	Danish Holstein (n=456) Danish Jersey (n=435) Swedish Red (n=407)	Poulsen et al. (2012)
Interbreed differences in coagulation properties (curd firming rate and rennet coagulation time), occurrence of non-coagulation and variant frequencies of the *CSN1S1*, *CSN2* and *CSN3* genes.	Danish Holstein (n=456) Danish Jersey (n=436) Swedish Red (n=407)	Poulsen et al. (2013)
Genetic parameter estimation and GWAS on milk riboflavin (vitamin B2) content.	Danish Holstein (n=456) Danish Jersey (n=436)	Poulsen et al. (2015a)
Heritability estimation and phenotypic association of milk compositional traits to rennet induced milk coagulation properties. Effect of somatic cell count on milk composition and milk coagulation properties.	Danish Holstein (n=456) Danish Jersey (n=435)	Poulsen et al. (2015c)
Factors influencing posttranslational modifications of caseins including association to rennet coagulation properties.	Danish Holstein (n=452) Danish Jersey (n=434)	Poulsen et al. (2016a)
Identification of β-casein variants by LC-ESI/MS in modern and native breeds and influence of genetic variants on β-casein content	Danish Holstein (n = 455) Danish Jersey (n = 433) Danish Red anno 1970 (n = 28) Jutland (n = 12)	Poulsen et al. (2016b)
Novel genetic variation associated to CSN3 affects rennet-induced milk coagulation	Danish Holstein (n=71)	Poulsen et al. (2017)
GWAS and heritabilities of bovine milk oligosaccharides	Danish Holstein (n=334) Danish Jersey (n=300)	Poulsen et al. (2019)
Aminoxy tandem mass tags for multiplexed milk oligosaccharide analysis	Method paper	Robinson et al. (2018)
Relative quantification of 13 milk oligosaccharides using aminoxyTMT.	Danish Holstein (n=334) Danish Jersey (n=300)	Robinson et al. (2019)
Metabolites related to milk coagulation (citrate, choline, carnitine and lactose) and breed (carnitine and lactose).	Danish Holstein (n=8) Danish Jersey (n=6)	Sundekilde et al. (2011)
Variability of bovine milk oligosaccharides.	Danish Holstein (n=10) Danish Jersey (n=10)	Sundekilde et al. (2012)
Association between milk somatic cell count and metabolites.	Danish Holstein-Friesian (n=456) Danish Jersey (n=436)	Sundekilde et al. (2013b)
NMR based metabolomics	Review	Sundekilde et al. (2013a)
Milk clotting properties, cheese yield, variation in milk protein composition (α-lactoglobulin, β-lactalbumin, caseins including the different genotypes).	Danish Holstein-Friesian (n=14) Swedish Red and white (n=16) Swedish Holstein (n=15)	Wedholm et al. (2006)
Use of infrared spectroscopy for quantification of orotic acid in milk and heritability estimation from this on larger study populations	Danish Holstein (n=673) Danish Jersey (n=351)	Zaalberg et al. (2020)

About DCA

DCA - Danish Centre for Food and Agriculture is the entrance to research in food and agriculture at Aarhus University (AU).

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SUMMARY

This report presents results from the Danish-Swedish Milk Genomics Initiative relating to the Danish animals, represented by Danish Holstein and Danish Jersey. The report summarizes results from more than 40 peer-reviewed papers published from the project initiative. The results relate to both new and updated measurements of compositional traits of bovine milk from individual cows sampled as part of the project The updated measurements regarding milk composition relates to content and variation of protein, fat, lactose, cell count, as well as more detailed parameters, like protein composition including genetic variants and their modified forms, metabolites, oligosaccharides, fatty acid composition, as well as major vitamins and minerals. Furthermore, processing characteristics like coagulation properties were central part of the studies, in addition to other nutritional and health related characteristics. The compositional traits were linked to genetic background, and potentials for breeding of improved milk composition relative to exploitation into dairy products is covered.